serotonin 5-HT6 receptors

(Updated 3rd or 4th quarter 2002)

Dawson LA, Nguyen HQ, Li P.

The 5-HT(6) receptor antagonist SB-271046 selectively enhances excitatory neurotransmission in the rat frontal cortex and hippocampus.

Neuropsychopharmacology 2001 Nov;25(5):662-8

"Preclinical evidence has suggested a possible role for the 5-HT(6) receptor in the treatment of cognitive dysfunction. However, currently there is little neurochemical evidence suggesting the mechanism(s) which may be involved. Using the selective 5-HT(6) antagonist SB-271046 and in vivo microdialysis, we have evaluated the effects of this compound on the modulation of basal neurotransmitter release within multiple brain regions of the freely moving rat. SB-271046 produced no change in basal levels of dopamine (DA), norepinephrine (NE) or 5-HT in the striatum, frontal cortex, dorsal hippocampus or nucleus accumbens. Similarly, this compound had no effect on excitatory neurotransmission in the striatum or nucleus accumbens. Conversely, SB-271046 produced 3- and 2-fold increases in extracellular glutamate levels in both frontal cortex and dorsal hippocampus, respectively. These effects were completely attenuated by infusion of tetrodotoxin but unaffected by the muscarinic antagonist, atropine. Here we demonstrate for the first time the selective enhancement of excitatory neurotransmission by SB-271046 in those brain regions implicated in cognitive and memory function, and provide mechanistic evidence in support of a possible therapeutic role for 5-HT(6) receptor antagonists in the treatment of cognitive and memory dysfunction." [Abstract]

Gerard C, Martres MP, Lefevre K, Miquel MC, Verge D, Lanfumey L, Doucet E, Hamon M, el Mestikawy S.

Immuno-localization of serotonin 5-HT6 receptor-like material in the rat central nervous system.

Brain Res 1997 Jan 23;746(1-2):207-19

Immunoautoradiographic experiments with antibodies affinity-purified on Affi-Gel coupled to the peptide antigen showed that 5-HT6-like immunoreactive material was abundant in the olfactory tubercle (plexiform layer), cerebral cortex (frontal and entorhinal areas), nucleus accumbens, striatum, hippocampus (strata oriens and radiatum of the CA1 area, molecular layer of the dentate gyrus) and the molecular layer of the cerebellum. A specific immunolabeling, but at moderate intensity, was also observed in the thalamus, substantia nigra, superficial layer of the superior colliculus, motor trigeminal nucleus and facial nucleus. In contrast, no 5-HT6-like immunoreactive material was found in white matter areas. As the regional distribution of 5-HT6 receptor-like immunoreactivity matched generally that previously found for the 5-HT6 receptor mRNA, one could infer that this receptor protein is addressed in the vicinity of its synthesis site, i.e. on somas and/or dendrites. Indeed, immunohistochemistry at the light and electron microscope level showed that 5-HT6-like immunoreactivity was associated with

Svenningsson P, Tzavara ET, Liu F, Fienberg AA, Nomikos GG, Greengard P.

DARPP-32 mediates serotonergic neurotransmission in the forebrain.

Proc Natl Acad Sci U S A 2002 Mar 5;99(5):3188-93

"Here we report that serotonin regulates DARPP-32 phosphorylation both in vitro and in vivo. Stimulation of 5-hydroxy-tryptamine (5-HT4 and 5-HT6) receptors causes an increased phosphorylation state at Thr34–DARPP-32, the protein kinase A site, and a decreased phosphorylation state at Thr75–DARPP-32, the cyclin-dependent kinase 5 site. Furthermore, stimulation of 5-HT2 receptors increases the phosphorylation state of Ser137–DARPP-32, the casein kinase-1 site. Behavioral and gene transcriptional effects induced by compounds that selectively release serotonin were greatly reduced in DARPP-32 knockout mice." [Full Text]

Boess, Frank G., Riemer, Claus, Bos, Michael, Bentley, Jane, Bourson, Anne, Sleight, Andrew J.

The 5-Hydroxytryptamine6 Receptor-Selective radioligand [3H]Ro 63-0563 Labels 5-Hydroxytryptamine Receptor Binding Sites in Rat and Porcine Striatum

Mol Pharmacol 1998 54: 577-583 [Full Text]

Miguel-Hidalgo JJ.

SB-271046 (SmithKline Beecham).

Curr Opin Investig Drugs 2001 Jan;2(1):118-22

"Data recently presented at the Society for Neuroscience annual meeting in November 2000 demonstrated that administration of SB-271046 resulted in a signficant increase in glutamate and aspartate levels in the frontal cortex, without affecting noradrenaline, dopamine or 5-HT levels. This was stated to suggest that 5-HT6 antagonists might therefore be useful for treating cognitive dysfunction [390469]." [Abstract]

Woolley ML, Bentley JC, Sleight AJ, Marsden CA, Fone KC.

A role for 5-ht6 receptors in retention of spatial learning in the Morris water maze.

Neuropharmacology 2001 Aug;41(2):210-9

"This study investigates the effect of intracerebroventricular administration of a 5-ht6 antisense oligonucleotide (AO) complementary to bases 1-18 of the rat 5-ht6 cDNA initiation sequence (Mol. Pharmacol. 43 (1993) 320) (1.5 microg twice daily for six days) and i.p. injection of a selective 5-ht6 receptor antagonist Ro 04-6790 (10 or 30 mg/kg once daily for three days) on acquisition and retention in the Morris water maze. Neither the 5-ht6 AO (which reduced cortical [3H]-LSD binding sites by 10-16%) nor Ro 04-6790 affected acquisition,

dendritic processes in both the striatum and the dentate gyrus of the hippocampus. The relative abundance of 5-HT6 receptor-like immunoreactivity in extrapyramidal and limbic areas suggests that 5-HT6 receptors may participate in the serotoninergic control of motor function and mood-dependent behavior, respectively." [Abstract]

Lauren P. Baker, Mark D. Nielsen, Soren Impey, Mark A. Metcalf, Steven W. Poser, Guy Chan, Karl Obrietan, Mark W. Hamblin, and Daniel R. Storm

Stimulation of Type 1 and Type 8 Ca2+/Calmodulin-sensitive Adenylyl Cyclases by the Gs-coupled 5-Hydroxytryptamine Subtype 5-HT7A Receptor

J. Biol. Chem. 273: 17469-17476, July 1998.

"In summary, these data demonstrate that 5-HT6 acts as a typical Gs-coupled receptor by stimulating AC5, but not AC1 or AC8. The discovery that 5-HT7A stimulates AC1 and AC8 through increases in intracellular Ca2+ provides a novel mechanism for serotonergic regulation of intracellular cAMP in the brain and other tissues." [Full Text]

East SZ, Burnet PW, Leslie RA, Roberts JC, Harrison PJ.

5-HT6 receptor binding sites in schizophrenia and following antipsychotic drug administration: Autoradiographic studies with [125I]SB-258585.

Synapse 2002 Sep;45(3):191-9

"In summary, [(125)I]SB-258585 is a suitable radioligand for studies of human brain 5-HT(6)R binding sites and shows that their distribution is broadly similar to that of the rodent. The lack of effect of schizophrenia or antipsychotic drug administration on [(125)I]SB-258585 binding suggests that an altered receptor density does not contribute to any involvement which the 5-HT(6)R may have in the disease or its treatment." [Abstract]

Masellis M, Basile VS, Meltzer HY, Lieberman JA, Sevy S, Goldman DA, Hamblin MW, Macciardi FM, Kennedy JL.

Lack of association between the T-->C 267 serotonin 5-HT6 receptor gene (HTR6) polymorphism and prediction of response to clozapine in schizophrenia.

Schizophr Res 2001 Jan 15;47(1):49-58 [Abstract]

Ohmori O, Shinkai T, Hori H, Nakamura J.

Novel polymorphism in the 5'-upstream region of the human 5-HT6 receptor gene and schizophrenia.

Neurosci Lett 2001 Sep 7;310(1):17-20

"Our results suggest that the 5-HT6 receptor gene polymorphism does not confer increased susceptibility to schizophrenia." [Abstract]

Shinkai T, Ohmori O, Kojima H, Terao T, Suzuki T, Abe K.

Association study of the 5-HT6 receptor gene in schizophrenia.

Am J Med Genet 1999 Apr 16;88(2):120-2

"No significant positive association between the 5-HT6 receptor genotype and schizophrenia was observed. Our results suggests that the 267C/T polymorphism of the 5-HT6 receptor gene may not be involved in the susceptibility to schizophrenia." [Abstract]

Rogers DC, Hagan JJ.

5-HT6 receptor antagonists enhance retention of a water maze task in the rat.

but both enhanced retention of the learned platform position such that rats spent significantly longer searching the trained platform position than any other area during the probe tests. Furthermore, neither AO nor Ro 04-6790 had any effect on the time taken to reach a raised visible platform, indicating that visual acuity was unimpaired. In addition, AO reduced both food consumption and body weight and the later effect was also seen following Ro 04-6790, suggesting a role for the 5-ht6 receptor in the regulation of feeding. Hence, while the underlying mechanism remains unclear, enhanced retention of spatial learning following both AO and 5-ht6 antagonist administration strongly indicate a role for this receptor in memory processes." [Abstract]

Stefulj J, Jernej B, Cicin-Sain L, Rinner I, Schauenstein K.

mRNA expression of serotonin receptors in cells of the immune tissues of the rat.

Brain Behav Immun 2000 Sep;14(3):219-24

"All 13 rat 5-HT receptor genes cloned so far were examined in ex vivo isolated spleen, thymus, and peripheral blood lymphocytes, as well as in mitogen-stimulated spleen cells. Positive signals were obtained for 5-HT1B, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT6, and 5-HT7 receptor mRNAs in all three compartments. Mitogen (ConA and PWM) stimulated cells additionally expressed mRNA corresponding to the 5HT-3 receptor subtype. In contrast, 5-HT1A, 5-HT1D, 5-HT2C, 5-HT4, 5-HT5A, and 5-HT5B mRNAs were not detected in any of the examined cell populations." [Abstract]

Branchek, Theresa A., Blackburn, Thomas P.

5-ht6 Receptors as Emerging Targets for Drug Discovery

Annu. Rev. Pharmacol. Toxicol. 2000 40: 319-334

"Surprisingly, 5-ht6 receptors appear to regulate cholinergic neurotransmission in the brain, rather than the expected interaction as modulators of dopaminergic transmission. This interaction predicts a possible role for 5-ht6 receptor antagonists in the treatment of learning and memory disorders." [Abstract]

Tsai SJ, Liu HC, Liu TY, Wang YC, Hong CJ.

Association analysis of the 5-HT6 receptor polymorphism C267T in Alzheimer's disease.

Neurosci Lett 1999 Dec 3;276(2):138-9

"Statistical analysis showed a significant difference in the genotype and gene frequencies between the AD group and the normal controls ($P = 0.006$; and $P = 0.023$, respectively). These findings indicate that the 267C allele of the 5-HT6 gene is a risk factor for AD." [Abstract]

Hamon M, Doucet E, Lefevre K, Miquel MC, Lanfumey L, Insausti R, Frechilla D, Del Rio J, Verge D.

Antibodies and antisense oligonucleotide for probing the distribution and putative functions of central 5-HT6 receptors.

Neuropsychopharmacology 1999 Aug;21(2 Suppl):68S-76S

"Studies using polyclonal anti-5-HT6 receptor antibodies and an antisense oligonucleotide were performed in order to investigate further the function(s) of 5-HT6 receptors in the rat brain. Immunocytochemistry at the light and electron microscope levels showed that 5-HT6 receptors are mainly confined to the dendritic compartment, suggesting that they could mediate 5-HT actions on neuronal firing. In some limbic

Psychopharmacology (Berl) 2001 Nov;158(2):114-9
" In the water maze, administration of SB-271046-A or SB-357134-A (3 or 10 mg/kg) had no effect on learning per se. At 10 mg/kg, however, both compounds produced a significant improvement in retention of a previously learned platform position when tested 7 days after training. By contrast, the acetylcholinesterase inhibitor, Aricept (donepezil, 0.1, 0.3 mg/kg PO) had no effect in this task." [Abstract]

Kohen R, Fashingbauer LA, Heidmann DE, Guthrie CR, Hamblin MW.
Cloning of the mouse 5-HT6 serotonin receptor and mutagenesis studies of the third cytoplasmic loop.
Brain Res Mol Brain Res 2001 Jun 20;90(2):110-7
"These data suggest that constitutive activity may be important to 5-HT6 receptor activity in vivo and that, unlike some other G-protein coupled receptors, alteration in the BBXXB CIII-loop motif reduces rather than further activates basal activity of the murine 5-HT6 receptor." [Abstract]

Matsumoto M, Yoshioka M.
[Possible involvement of serotonin receptors in anxiety disorders]
Nippon Yakurigaku Zasshi 2000 Jan;115(1):39-44
"Inactivation of mRNA encoding 5-HT6 receptors using antisense oligonucleotide produced decreases in cortical 5-HT release enhanced by anxiety. These observations lead to the suggestion that different mechanisms, mediated by various 5-HT receptors, are involved in the pathogenesis of anxiety." [Abstract]

Healy DJ, Meador-Woodruff JH.
Ionotropic glutamate receptor modulation of 5-HT6 and 5-HT7 mRNA expression in rat brain.
Neuropsychopharmacology 1999 Sep;21(3):341-51
"MK-801 treatment induced a dose-dependent decrease in striatal 5-HT6 receptor mRNA levels; similarly, both aniracetam and NBQX treatments also led to decreases in striatal 5-HT6 receptor mRNA levels. Hippocampal 5-HT6 and 5-HT7 receptor expression were not dramatically affected by any of the treatments. To our knowledge, this is the first demonstration of the regulation of striatal 5-HT6 receptor mRNA expression, and provides neurochemical anatomical evidence for the interaction of serotonergic and glutamatergic systems."
[Abstract]

Pena-Rangel MT, Mercado R, Hernandez-Rodriguez J.
Regulation of glial Na+/K+-ATPase by serotonin: identification of participating receptors.
Neurochem Res 1999 May;24(5):643-9
"Altogether, these results show that serotonin modulates glial Na+/K+-ATPase activity in the brain, apparently not through only one type of 5-HT receptor. It seems that the receptor system involved is different according to the brain region. In cerebral cortex, the response seems to be mediated by 5-HT1A as well as in hippocampus but not in cerebellum where 5-HT6 appears as the receptor system involved." [Abstract]

Bentley, Jane C., Bourson, Anne, Boess, Frank G., Fone, Kevin C.F., Marsden, Charles A., Petit, Nadine, Sleight, Andrew J.
Investigation of stretching behaviour induced by the selective 5-HT6 receptor antagonist, Ro 04-6790, in rats
areas, 5-HT6 receptor-like immunoreactivity is also associated with neuronal cilia with yet unknown functions. Continuous i.c.v. infusion with an antisense oligonucleotide for 3-4 days resulted in decreased 5-HT6 receptor-like immunostaining of the nucleus accumbens and anxiogenic behaviours in the social interaction and elevated plus maze tests." [Abstract]

Routledge, Carol, Bromidge, Steven M., Moss, Stephen F., Price, Gary W., Hirst, Warren, Newman, Helen, Riley, Graham, Gager, Tracey, Stean, Tania, Upton, Neil, Clarke, Stephen E., Brown, Anthony M., Middlemiss, Derek N.
Characterization of SB-271046: A potent, selective and orally active 5-HT6 receptor antagonist
Br. J. Pharmacol. 2000 130: 1606-1612
"SB-271046 produced an increase in seizure threshold over a wide-dose range in the rat maximal electroshock seizure threshold (MEST) test, with a minimum effective dose of 0.1 mg kg-1 p.o. and maximum effect at 4 h post-dose. The level of anticonvulsant activity achieved correlated well with the blood concentrations of SB-271046 (EC50 of 0.16 μM) and brain concentrations of 0.01 – 0.04 μM at Cmax.

These data, together with the observed anticonvulsant activity of other selective 5-HT6 receptor antagonists, SB-258510 (10 mg kg-1, 2 – 6 h pre-test) and Ro 04-6790 (1 – 30 mg kg-1, 1 h pre-test), in the rat MEST test, suggest that the anticonvulsant properties of SB-271046 are likely to be mediated by 5-HT6 receptors." [Abstract]

Boess FG, Monsma FJ Jr, Sleight AJ.
Identification of residues in transmembrane regions III and VI that contribute to the ligand binding site of the serotonin 5-HT6 receptor.
J Neurochem 1998 Nov;71(5):2169-77
"We have examined the ligand binding site of the serotonin 5-HT6 receptor using site-directed mutagenesis. Replacing the highly conserved Asp106 in transmembrane region III by asparagine eliminated D-[3H]-lysergic acid diethylamide ([3H] LSD) binding to the mutant receptor transiently expressed in HEK293 cells. The potency of 5-HT and LSD to stimulate adenylyl cyclase was reduced by 3,600- and 500-fold, respectively, suggesting that an ionic interaction between the positively charged amino group of 5-HT and D106 is essential for high-affinity binding and important for receptor activation. In addition, basal cyclic AMP levels in cells expressing this mutant were increased. Mutation of a tryptophan residue one helix turn toward the extracellular side of transmembrane region III (Trp102) to phenylalanine produced significant changes in the binding affinity and potency of several ligands, consistent with a role of this residue in the formation of the ligand binding site. The exchange of two neighboring residues in the carboxy-terminal half of transmembrane region VI (Ala287 and Asn288) for leucine and serine resulted in a mutant receptor with increased affinities (seven- to 30-fold) for sumatriptan and several ergopeptine ligands. The identification of these interactions will help to improve models of the 5-HT6 receptor ligand binding site." [Abstract]

Boess, Frank G., Monsma, Frederick J., Jr., Meyer, Valerie, Zwingelstein, Catherine, Sleight, Andrew J.
Interaction of Tryptamine and Ergoline

Br. J. Pharmacol. 1999 126: 1537-1542
"These data suggest that systemic injection of the 5-HT6 antagonist, Ro 04-6790, produces a stretching behaviour that appears to be mediated by an increase in cholinergic neurotransmission in the CNS and which could be a useful functional correlate for 5-HT6 receptor blockade. There is no evidence for dopamine D2-like receptor involvement in this behaviour." [Abstract]

Bourson, A, Boess, FG, Bos, M, Sleight, AJ

Involvement of 5-HT6 receptors in nigro-striatal function in rodents

Br. J. Pharmacol. 1998 125: 1562-1566
"Ro 04-6790 (3, 10 and 30 mg kg(-1) i.p.) did not itself induce rotational behaviour in rats with unilateral 6- hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle nor did it affect the rotational behaviour induced by either L-Dopa or amphetamine. 5-HT6 receptor antagonism inhibited the rotational behaviour of 6-OHDA lesioned rats induced by treatment with the muscarinic antagonists scopolamine and atropine. The data support earlier conclusions from experiments with antisense oligonucleotides that the 5-HT6 receptor is involved in the control of acetylcholine neurotransmission in the rat brain." [Abstract]

Hirst, Warren D., Minton, Jayne A.L., Bromidge, Steven M., Moss, Stephen F., Latter, Alison J., Riley, Graham, Routledge, Carol, Middlemiss, Derek N., Price, Gary W.

Characterization of [125I]-SB-258585 binding to human recombinant and native 5-HT6 receptors in rat, pig and human brain tissue

Br. J. Pharmacol. 2000 130: 1597-1605
"The pKi rank order of potency for a number of compounds, determined in competition binding assays with [125I]-SB-258585, at human caudate putamen membranes was: SB-271046>SB-258585>SB-214111>methiothepin>clozapine>5-Me-OT>5-HT>Ro 04-6790>mianserin>ritanserin=amitriptyline>5-CT>mesulergine. Similar profiles were obtained from pig and rat striatal membranes and recombinant 5-HT6 receptors; data from the latter correlated well with [3H]-LSD binding." [Abstract]

Purohit A, Herrick-Davis K, Teitler M.

Creation, expression, and characterization of a constitutively active mutant of the human serotonin 5-HT6 receptor.

Synapse 2003 Mar;47(3):218-24
"The serotonin 5-HT(6) receptor, a G-protein-coupled receptor, displays high affinity for antipsychotic, antidepressant, and psychotropic drugs. We created a constitutively active form of the human 5-HT(6) receptor in order to probe the molecular domains of receptor activation and to determine if inverse agonist activities of antipsychotic drugs contribute to their clinical profile. Previous studies from our laboratory support a critical role for the c-terminal region of the third intracellular loop (il3) in the activation of G(q)-coupled serotonin receptors. In the present study, PCR-based mutagenesis was used to mutate serine 267 (S6.34) in the c-terminal region of il3 to lysine (S267K). The native and S267K 5-HT(6) receptors were expressed in COS-7 cells to study the functional effects of the mutation. The S267K receptor shows 10-fold higher affinity for serotonin than the native receptor and demonstrates agonist-independent activity. Clozapine decreased the basal activity of the S267K receptor to vector control levels. Therefore, we can conclude that the S267K mutation renders the 5-HT(6) receptor constitutively active and that clozapine is an inverse agonist at the mutant 5-HT(6) receptor. These results indicate that the c-terminal

Compounds with Threonine 196 in the Ligand Binding Site of the 5-Hydroxytryptamine6 Receptor

Mol Pharmacol 1997 52: 515-523
"The combination of site-directed mutagenesis (guided by knowledge obtained for related receptors) with a series of related ligands differing in a particular structural feature has allowed the identification of a specific interaction between Thr196 in transmembrane region V of the 5-HT6 receptor and the indole nitrogen of N1-unsubstituted ergolines and tryptamines." [Full Text]

Sleight AJ, Monsma FJ Jr, Borroni E, Austin RH, Bourson A.

Effects of altered 5-ht6 expression in the rat: functional studies using antisense oligonucleotides.

Behav Brain Res 1996;73(1-2):245-8
"Rats were treated with either saline, antisense (AO) or scrambled oligonucleotides (SO) for 4 days. Treatment with AO reduced the number of [3H]LSD binding sites in the frontal lobes by 30% but had no significant effect on the number of 5-HT1A and 5-HT2A receptor binding sites in the cortex of the rats. A behavioural syndrome of yawning, stretching and chewing, however, was observed in AO treated rats but not in any of the other treatment groups. This AO-specific behaviour had returned to normal 5 days after cessation of the oligodeoxynucleotide treatment." [Abstract]

Kohen R, Metcalf MA, Khan N, Druck T, Huebner K, Lachowicz JE, Meltzer HY, Sibley DR, Roth BL, Hamblin MW.

Cloning, characterization, and chromosomal localization of a human 5-HT6 serotonin receptor.

J Neurochem 1996 Jan;66(1):47-56
"The human 5-HT6 amino acid sequence is 89% similar to the corrected rat sequence. The recombinant human 5-HT6 receptor is positively coupled to adenylyl cyclase and has pharmacological properties similar to the rat receptor with high affinity for several typical and atypical antipsychotics, including clozapine. The receptor is expressed in several human brain regions, most prominently in the caudate nucleus. The gene for the receptor maps to the human chromosome region 1p35-p36. This localization overlaps that established for the serotonin 5-HT1D alpha receptor, suggesting that these may be closely linked." [Abstract]

region of il3 of the G(s)-coupled 5-HT(6) receptor is a key domain for G-protein coupling, similar to the G(q)-coupled 5-HT receptors. The inverse agonist action of clozapine indicates that drugs displaying competitive antagonist activity at native 5-HT(6) receptors may display inverse agonist activity at the constitutively activated form of the receptor." [Abstract]

Olsen MA, Nawoschik SP, Schurman BR, Schmitt HL, Burno M, Smith DL, Schechter LE.

Identification of a human 5-HT6 receptor variant produced by alternative splicing.

Brain Res Mol Brain Res 1999 Feb 5;64(2):255-63

"The splicing pattern seen for this transcript was not detected in rat or mouse whole brain cDNA by PCR due to the lack of a consensus 5' donor site. Coexpression of the variant 5-HT6 transcript and the full length 5-HT6 transcript was observed in caudate and substantia nigra but not in hippocampus, cortex, cerebellum and thalamus. Transient transfection of a 5-HT6 variant construct into Cos-7 cells demonstrated that a truncated receptor was translocated to the membrane but appeared nonfunctional." [Abstract]

Yau JL, Noble J, Widdowson J, Seckl JR.

Impact of adrenalectomy on 5-HT6 and 5-HT7 receptor gene expression in the rat hippocampus.

Brain Res Mol Brain Res 1997 Apr;45(1):182-6

"Here, we show that pharmacological adrenalectomy increases 5-HT6 and 5-HT7 receptor mRNA expression in specific hippocampal subfields, effects partly reversed by corticosterone replacement." [Abstract]

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An Assessment of the Effects of 5-HT6 Receptor Antagonists in Rodent Models of Learning

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Non-standard abbreviations: serotonin 6 (5-HT6)

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ABSTRACT

Antagonists of serotonin 6 (5-HT6) receptors have been reported to enhance cognition in animal models of learning, although this finding has not been universal. We have assessed the therapeutic potential of the specific 5-HT6 receptor antagonists, Ro 04-6790 and SB-271046, in rodent models of cognitive function. Although mice express the 5-HT6 receptor and the function of this receptor has been investigated in mice, all reports of activity with 5-HT6 receptor antagonists have utilized rat models. In the present study, receptor binding revealed that the pharmacological properties of the mouse receptor are different from the rat and human receptor: Ro 04-6790 does not bind to the mouse 5-HT6 receptor, so all in vivo testing included in the present report was conducted in rats. We replicated previous reports that 5-HT6 receptor antagonists produce a stretching syndrome previously shown to be mediated through cholinergic mechanisms, but Ro 04-6790 and SB-271046 failed to attenuate scopolamine-induced deficits in a test of contextual fear conditioning. We also failed to replicate the significant effects reported previously in both an autoshaping task and in a version of the Morris water maze. The results of our experiments are not consistent with previous reports that suggested that 5HT6 antagonists might have therapeutic potential for cognitive disorders. In our experiments, reference 5-HT6 receptor antagonists failed to demonstrate any significant effects suggestive of utility as cognition enhancing agents.

The 5-HT6 receptor was first isolated from rat striatal mRNA in 1993. It is localized almost exclusively in the CNS, including areas important for learning and memory, such as the cerebral cortex and hippocampus (Monsma, Jr. et al., 1993; Ruat et al., 1993). Polymorphisms of the 5-HT6 receptor have been associated with clinical disorders such as Alzheimer's, bipolar affective disorder, and schizophrenia (Tsai et al., 1999a; Tsai et al., 1999b; Vogt et al., 2000), all of which are characterized by at least some degree of cognitive deficit. The suggestion that 5-HT6 receptor antagonists may have therapeutic potential as novel treatments for cognitive deficits is supported by reports that they facilitate cholinergic and glutamatergic neurotransmission. Antagonists of 5-HT6 receptors produce a behavioral syndrome of yawning/stretching/chewing, which is characteristic of cholinergic agonists (Bourson et al., 1995; Sleight et al., 1996; Sleight et al., 1998; Bentley et al., 1999), and they reduce the number of rotations produced in rats by cholinergic antagonists (Bourson et al., 1998). 5-HT6 receptor antagonists have also been shown to enhance extracellular levels of glutamate in the frontal cortex and hippocampus as revealed during microdialysis (Dawson et al., 2000; Dawson et al., 2001).

In addition, there is suggestive evidence that atypical anti-psychotics may attenuate cognitive deficits in patients with schizophrenia, perhaps through their action as 5-HT6 receptor antagonists. Atypical anti-psychotics have very high affinities for 5-HT6 receptors and block stimulation of adenylyl cyclase activity produced by serotonin (Sebben et al., 1994). They enhance extracellular levels of glutamate in the frontal cortex (Daly and Moghaddam, 1993), and chronic treatment with atypical anti-psychotics decreases 5-HT6 receptor expression in the hippocampus (Frederick and Meador-Woodruff, 1999). Most patients with schizophrenia have cognitive deficits (Meltzer and McGurk, 1999), and atypical anti-psychotics attenuate these cognitive deficits (Purdon et al., 2000). Typical anti-psychotics such as haloperidol do not mediate their effects through the 5-HT6 receptor (Bourson et al., 1995; Bourson et al., 1998; Bentley et al., 1999; Frederick and Meador-Woodruff, 1999), nor do they attenuate cognitive deficits in schizophrenia patients (Purdon et al., 2000). Taken together, these results may suggest that the cognitive effects of atypical anti-psychotics may be mediated by their action as 5-HT6 receptor antagonists.

Finally, several studies have reported that specific 5-HT6 receptor antagonists improve learning and memory in animal models. Analogues of the selective 5-HT6 receptor antagonist Ro 04-6790 attenuated scopolamine-induced deficits in a passive avoidance task (Bos et al., 2001). Ro 04-6790 also increased acquisition and consolidation in normal young rats in an operant autoshaping task, and it attenuated scopolamine-induced deficits in this task (Meneses, 2001). The selective 5-HT6 receptor antagonists Ro 04-6790, SB-271046 and SB-357134, all increased retention of a spatial mapping Morris water maze task in normal young rats (Woolley et al., 2001; Rogers and Hagan, 2001; Stean et al., 2002).

In contrast to all the studies which support the therapeutic potential of 5-HT6 receptor antagonists, Russell and Dias (2002) reported that they were unable to replicate any of the therapeutic effects of 5-HT6 receptor antagonists. The objective of the studies included in the present report was to assess the therapeutic potential of 5-HT6 receptor antagonists for enhancing cognitive function in rodent models. Both rats and mice express the 5-HT6 receptor, and both species have been used to investigate the function of this receptor. Initially, we planned to assess 5-HT6 receptor antagonists in both mouse and rat models of cognitive behavior. However, although mice have been used to assess the therapeutic potential of 5-HT6 receptor antagonists (Bourson et al., 1998), all positive effects with 5-HT6 receptor antagonists have been detected in rats, and our own initial work with mice failed to detect therapeutic effects with 5-HT6 receptor antagonists (data not shown). Examination of the literature revealed that four critical residues have been identified for ligand binding to the 5-HT6 receptor, and one of these four residues is different in the mouse receptor when compared with rat and human receptor (Boess et al., 1998; Kohen et al., 2001). Therefore, the objective of our first experiment was to determine if 5-HT6 receptor antagonists bind to the mouse receptor. This study revealed that the pharmacological properties of the mouse receptor are different from the rat and human receptor, so we subsequently conducted all efficacy tests in rats. In addition, since most of the published literature demonstrating the therapeutic potential of 5-HT6 receptor antagonists was produced with Ro 04-6790 or SB-271046, we focused our efforts on attempting to replicate the initial preclinical experiments using these two standards.

MATERIALS and METHODS

Animals. These studies were conducted in an animal care facility certified by the American Association for Accreditation of Laboratory Animal Care, and all experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee before the studies were initiated. All rats were from Harlan Sprague-Dawley (Indianapolis, IN): male Sprague-Dawley, 285 ± 1.8g, N=297; male hooded Long-Evans, 282 ± 4.8g, N=69; and male Wistar, 360 ± 4.7g, N=160. Rats were housed in polycarbonate cages in a temperature-controlled room with a 12:12hr light:dark cycle. All rats were housed 3-4 per cage, and food and water was available *ad libitum*, except for the Wistar rats that were used in the food reward autoshaping task, which were singly housed and maintained on a restricted diet, 12-15g per day of standard rat chow, until their body weights were approximately 85% of *ad lib* fed rats, at which time behavioral testing was initiated.

Cloning and transfection of human, rat and mouse 5-HT6 receptors. The mouse and rat 5HT-6 receptors were obtained by PCR amplification using whole rat (adult male Sprague-Dawley) or mouse (adult male BALB/c) brain cDNA (Clontech, Palo Alto, CA) followed by TA cloning, insert excision and purification, and finally cloning into the pCDNA3.1 vector (Invitrogen, Frederick, MD). The mouse insert was amplified using 0.4μM each of the following primers: 5'-ATGGTTCCAGAGCCCGGCCCTGTCAAC-3' and 5'-TCAGTTCATGGGGGAACCAAGTGGATGCTG-3'. The rat insert was amplified using 0.4μM each of the following primers: 5'- ATGGTTCCAGAGCCAGGCCCTGTCAAC-3' and 5'-CTCCAATGGCCAGCTCTTGACCTGGTCA-3'. The completed vectors were transformed into DH5αF competent cells (Invitrogen) and a large scale prep of DNA was prepared using the Qiafilter Plasmid Maxi Kit (Qiagen, Valencia, CA) for sequence confirmation followed by transfection. The human 5-HT6 receptor was obtained from David R. Sibley (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland). It was subcloned into pCDNA3.1 as well.

The constructs were transiently transfected into HEK 293 human embryonic kidney cells using Lipofectamine Plus (Invitrogen). Briefly, 1.5×10^7 cells were plated into 150mm tissue culture plates 24

hrs prior to transfection. Cells were transfected using 8μg DNA, 40μl plus reagent and 60μl lipofectamine reagent per plate according to manufacturer's instructions. Following a 3-hour transfection incubation, cells were fed fresh medium and were harvested 48 hours later. Control transfections with a fluorescent protein demonstrated an 80% transfection efficiency.

Cells were rinsed, scraped up and homogenized with a polytron. The cell homogenate was centrifuged for 30 minutes at 30,000 x g. The pellet was resuspended in 50mM Tris, pH 7.2, 1mM EDTA plus Sigma Mammalian Protease Inhibitor (St. Louis, MO). Protein concentration was determined using bicinchoninic acid (Pierce, Rockford, IL).

5HT-6 Receptor Binding Studies. The protocol was adapted from a previous report (Boess et al., 1997). The Bmax values for the human, rat, and mouse membrane preparations were human = 18,000 fmols/mg; rat=11,400 fmols/mg; mouse =4600 fmols/mg. The membranes (2, 5 and 15 μg protein for the human, rat, and mouse receptors respectively) were added to [^{3}H] d-lysergic acid diethylamide (LSD, 84 Ci/mmol, Amersham, Piscataway, NJ) to start the binding assay in 50 mM Tris, 2 mM $MgCl_2$, pH 7.4. The [^{3}H]LSD was diluted in 0.4% bovine serum albumen, 200 μM ascorbic acid and then diluted 1:10 in the samples to give a final concentration of 2 nM. Final concentrations of compounds ranged from 10^{-6} to 10^{-10} M and were dissolved in dimethylsulfoxide (DMSO) with a final concentration of 1% DMSO. Non-specific binding was determined using 10 μM clozapine. The 96 well plates were shaken for 10 minutes at room temperature on a Lab Line Instruments Titer Plate Shaker (Melrose Park, IL) and then incubated for 1 hour at 37°C. The samples were filtered through Whatman GF/B membranes pretreated with 0.5% polyethyleneimine. The samples were immediately washed 5 times with 1.5 ml ice cold 20 mM Tris, pH 7.4. They were counted in a Wallac Microbeta Trilux 1450 scintillation counter (Turku, Finland).

Yawning/stretching/chewing. Rats were given vehicle injections and placed in individual, transparent chambers for one hour each day for four days before the test day, to habituate them to the observation chambers and testing procedure. On the test day, rats were placed in the observation chambers immediately after drug administration and observed continuously for yawning, stretching and chewing

behaviors from 30-90 minutes after drug or vehicle injections (Ro 04-6790 30.0 mg/kg; SB-271046 30.0 mg/kg; physostigmine 0.1 mg/kg). Average number of yawns, stretches, and vacuous chewing movements during the one hour observation period were recorded as previously reported (Sleight et al., 1998; Bentley et al., 1999).

Conditioned Fear. Rats were first placed in individual sound-attenuating test chambers (Med Associates, St. Albans, VT) for a six minute conditioning session, which consisted of 2 minutes of habituation, a tone/footshock pairing (30 second 87 dB tone, 2 second 2.5 mA footshock), followed by 2 minutes with no tone or shock, another tone/footshock pairing, and a final minute of no stimuli presentation. Control rats were dosed with vehicle and placed in the fear conditioning box for 6 minutes but with no tone or shock. Rats were then removed from the test box and returned to their home cages. Percent time freezing was measured 24 hours later in a seven minute contextual memory test in the same chambers with no tone or shock. Freezing behavior was quantified with Freezeview image analysis software (Actimetrics, Evanston, IL) using filter 25, and bout length of 0.75 sec. SB-271046 solutions (1.0, 10.0, and 30.0 mg/kg, p.o.) and scopolamine (1.0 mg/kg, i.p.) were co-administered 1.5 hours before the conditioning session. Ro 04-6790 (30.0 mg/kg, i.p.) and scopolamine (1.0 mg/kg, i.p.) were co-administered 30 minutes before the conditioning session. All drugs were administered before the conditioning session only.

Autoshaping. Ro 04-6790 was tested in an autoshaping procedure as previously reported (Meneses, 2001). On the first day of testing rats were habituated to operant chambers (Coulbourn Instruments, Allentown, PA) by filling the food trough with 50 food pellets (45 mg/pellet). As soon as all the pellets were eaten, each rat was given 10 trials. During a trial, rats were presented with a retractable lever for 8 seconds and the cage was illuminated by a house light located at the top of the cage directly above the lever. After 8 seconds the lever was retracted, the house light was extinguished and a 45mg food pellet was delivered to the food trough. If the rat pressed the lever while it was extended, it was immediately retracted, the pellet was delivered and the light was extinguished. Following pellet delivery there was a 60s intertrial interval. The following day, each rat was tested by giving them 20 trials without the initial 50-pellet habituation period. Ro 04-6790 (0, 1, 5 or 10 mg/kg, IP, 1 ml/kg, in sterile saline) was administered

immediately after training, and scopolamine (0.17 mg/kg, IP) was administered 10 minutes after the training session, as previously reported (Meneses, 2001). The dependent measure was the percentage of bar-presses during the test session. In order to overcome potential floor effects, another experiment was conducted in which rats were given additional daily test sessions, first using the same procedures as above, and then under slightly different conditions to further facilitate the rate of acquisition, in which the lever remained extended for 30 seconds, and was retracted for 10 seconds, for 50 trials each day, as previously reported (Andrews et al., 1995).

Morris water maze. Ro 04-6790 was tested in the Morris water maze as previously reported (Woolley et al., 2001). Briefly, rats were administered saline vehicle or Ro 04-6790 at the optimal dose (30 mg/kg, IP, 1ml/kg) 30 minutes before daily acquisition training. During acquisition training rats received three, 90 second trials per day for 3 days, to swim to a hidden platform, with 20 seconds on the platform at the end of each trial. They were then given one probe trial per day, without drug, 7, 10 and 14 days after the end of acquisition training. During probe trials, the hidden platform was removed, rats were allowed to swim for 60 seconds, and swimming duration within a 10cm annulus around the former platform location was quantified. One experiment was conducted with Sprague-Dawley rats and another experiment was conducted with Long Evans Hooded Rats.

SB-271046 was also tested in the Morris water maze as previously reported (Rogers and Hagan, 2001). Briefly, rats were dosed with vehicle or SB-271046 at the optimal dose (10 mg/kg, PO, 2ml/kg in 1% methylcellulose) 2 hours before acquisition and probe test sessions. Rats were tested in four 60-second acquisition trials on day 1, and 6 acquisition trials per day on days 2-5. Probe trials were conducted immediately after the last acquisition trial, and again 4, 7 and 10 days after the end of acquisition training. Latency to reach the platform was recorded during acquisition trials, and the percent time in the target quadrant was the dependent measure during probe trials.

Drugs. Ro 04-6790 and SB-271046 were synthesized at Bristol-Myers Squibb (Wallingford, CT) and the structures confirmed using standard analytical methods. Scopolamine hydrobromide and physostigmine

salicylate were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in sterile saline and administered IP at 1ml/kg, except SB-271046 in the conditioned fear and Morris water maze experiments, where it was suspended in 1% methyl cellulose and administered by oral gavage at 1-2 ml/kg.

Statistics. Data analyses were conducted with SAS™. In the text and all figures, data are presented as means ± SEMs. The yawning, stretching and chewing, conditioned fear and autoshaping data were analyzed with planned contrasts between the main control group and each treatment group, including repeated measures where appropriate. The data in the last autoshaping experiment was analyzed with a 2 x 2 ANOVA, including Ro 04-6790 and Scopolamine as factors in the analysis. In the Morris water maze experiments, differences between groups were analyzed separately for each trial during acquisition, as previously reported (Woolley et al., 2001). For probe trials with SB-271046, potential differences between groups in duration of time spent swimming in the target quadrant was analyzed separately for each probe trial, as previously reported (Rogers and Hagan, 2001). For probe trials with Ro 04-6790, performance was analyzed separately for each group and for each probe trial: duration of time in each quadrant was compared to the duration of time spent swimming in the target quadrant, as previously reported (Woolley et al., 2001).

RESULTS

5-HT6 Receptor Binding Studies. Receptor binding studies were performed using recombinant receptors from human, rat, and mouse. Clozapine and methiothepin were shown to have similar Ki's at the 5-HT6 receptor from all 3 species. All the compounds tested, including SB-271046, Ro 04-6790 and clozapine, had potencies in the rat receptor that were similar to their potencies in the human receptors; however, a different pharmacology was observed for the mouse receptor (Fig. 1, Table 1). SB-271046 had a four-fold lower affinity at the mouse receptor than at the rat receptor, and while Ro 04-6790 had a Ki of 23.07 nM in rat receptors, it failed to inhibit LSD binding in the mouse receptor up to 1 μM (Table 1). We do not know if these changes are manifest in all mouse strains but based on these results, all subsequent in vivo experiments were conducted in rats.

Yawning/Stretching/Chewing. In an experiment with the optimal dose of SB-271046, physostigmine produced a significant increase in chewing behaviors, $F(1,29)=6.27$, $p=0.02$; and a trend towards increased yawning, $F(1,29)=2.19$, $p=0.15$. SB-271046 produced a significant increase in stretching behavior, $F(1,29)=6.55$, $p=0.01$ (Fig. 2). In an experiment examining the effects of the optimal dose of Ro 04-6790, physostigmine produced a significant increase in chewing, $F(1,21)=4.44$, $p=0.04$, and a trend towards increased yawning, $F(1,21)=3.21$, $p=0.09$; while Ro 04-6790 produced a significant increase in the number of stretches, $F(1,21)=8.72$, $p=0.008$, and vacuous chewing, $F(1,21)=4.44$, $p=0.04$ (Fig. 3).

Conditioned Fear. In the conditioned fear experiment with Ro 04-6790, there were significant differences between [1] the no-shock controls and the shocked, vehicle-treated controls, $F(1,75)=32.52$, $p=0.0001$, [2] the scopolamine-treated group and the vehicle group, especially during the later time points - the treatment x minute interaction between those groups was statistically significant, $F(6,450)=2.18$, $p=0.04$; [3] the no-shock controls versus the scopolamine-treated group, $F(1,75)=15.20$, $p=0.0002$; and [4] the Ro 04-6790 group versus the scopolamine treated group, $F(1,75)=5.58$, $p=0.02$ (Fig. 4A). Ro 04-6790 at 30 mg/kg reduced the amount of freezing beyond that seen in animals treated with scopolamine only, an effect which is consistent with impaired performance.

In the conditioned fear experiment with SB-271046, there were significant differences between [1] the no-shock controls and the shocked, vehicle-treated controls, $F(1,126)=51.65$, $p=0.0001$, [2] the scopolamine-treated group and the vehicle group, $F(1,126)=5.18$, $p=0.02$; [3] the no-shock controls and the scopolamine-treated group, $F(1,126)=24.66$, $p=0.0001$; and [4] the SB-271046 30mg/kg treated group and the scopolamine-treated group, $F(1,126)=6.93$, $p=0.01$ (Fig. 4B). Again, the effect of SB-271046 at 30 mg/kg was in the direction of impaired performance, relative to the group treated with scopolamine alone. Lower doses of SB-271046 did not produce significant effects, but even at 1.0 and 10.0 mg/kg, the trend was in the direction of impaired performance, relative to animals treated with scopolamine alone.

Autoshaping. In the first autoshaping experiment with Ro 04-6790, there were no significant increases in bar-pressing in animals with any dose of Ro 04-6790, $F's(1,73)<1.27$, $p's > 0.26$ (Fig. 5A). In the second autoshaping experiment examining the ability of Ro 04-6790 to attenuate the effects of scopolamine, dosing rats with 0.17 mg/kg scopolamine after the first session did not significantly reduce bar-pressing 24 hours later, and Ro 04-6790 did not increase bar-pressing rate on its own or in animals dosed with scopolamine, $F's(1,43)< 1.0$, $p's > 0.40$ (Fig. 5B). In the third autoshaping experiment, rats were repeatedly tested with scopolamine (0.17 mg/kg) and Ro 04-6790 administered after each session, using the dose of Ro 04-6790 (5 mg/kg) that previously produced peak effects (Meneses, 2001). In order to facilitate acquisition of bar-pressing and overcome any potential floor effects, an additional session was conducted using the Meneses procedure, and 5 more sessions with 50 trials per day, with the duration of lever extension increased from 8 seconds to 30 seconds. Rats did begin to acquire this task - bar-pressing increased over days, and a scopolamine deficit was eventually evident, the day x scopolamine interaction was statistically significant, $F(6,318)=2.34$, $p=0.03$ (Fig. 6). Ro 04-6790 (5mg/kg) did not facilitate acquisition or attenuate the scopolamine-related deficit, in fact, there was a trend for Ro 04-6790 to impair performance in both vehicle and scopolamine-treated rats, but that trend was not significant.

Morris Water Maze. In the test of Ro 04-6790 in both albino Sprague-Dawley and hooded Long-Evans rats, latencies to reach the target platform during acquisition trials improved over trials (Fig. 7). In the

albino Sprague-Dawley rats, there was a significant difference between the vehicle and Ro 04-6790 only on the second trial of acquisition training, in which the drug-treatment group had longer latencies than the vehicle-treated group, $F(1,28)=6.79$, $p=0.01$ (Fig. 7A). In the hooded Long-Evans rats, there was a significant difference between the vehicle and Ro 04-6790 only on the fourth trial of acquisition training, in which the drug-treatment group had longer latencies than the vehicle-treated group, $F(1,27)=6.89$, $p=0.01$ (Fig. 7B).

Analyses of probe trial data revealed no evidence that Ro 04-6790 increased retention of the target location in either albino Sprague-Dawley rats or hooded Long-Evans rats (Fig. 8). Although the hooded Long-Evans rats had more of a preference for the target annulus during the first probe trial, both albino Sprague-Dawley rats and hooded Long-Evans rats spent more time swimming in the target annulus on the first probe trial, 7 days after the end of acquisition training, regardless of whether they were in the vehicle or Ro 04-6790 group. The Ro 04-6790-treated Sprague-Dawleys may not have had quite as strong a preference for the target location as the vehicle-treated rats during the first probe trial, since they failed to discriminate between the target quadrant and one of the adjacent quadrants (Fig. 8A, top left panel). In the second probe trial, 10 days after the end of acquisition training, vehicle-treated Sprague-Dawley rats had no preference for the target annulus, while the Ro 04-6790-treated rats swam in the target annulus more than in one of the adjacent quadrants, but the trend was just the opposite in hooded Long-Evans rats, where only the vehicle-treated group swam more in the target annulus than in one of the adjacent quadrants. On the third probe trial, 14 days after the end of acquisition training, there was no preference to swim in the target annulus in any of the groups. In fact, the hooded Long-Evans rats treated with Ro 04-6790 actually exhibited a preference to swim in quadrants other than where the target had been located (Fig. 8B, bottom right panel).

Hooded Long-Evans rats rapidly acquired the Morris water maze task to asymptotic levels of performance, but SB-271046 did not affect latencies to find the Morris water maze, $F's(1,38)<1.4$, $p's>0.25$ (Fig. 9A). Rats also exhibited evidence that they were utilizing spatial-mapping strategies, since they swam almost 50% of the time in the target quadrant during the first probe trial immediately after the

last acquisition trial (Fig. 9B). The spatial mapping strategy extinguished over repeated probe trials, as the rats spent less and less time swimming near the former target location. There was a trend for the vehicle-treated rats to spend more time swimming in the target quadrant on the third probe trial, and a trend for the SB-271046-treated rats to spend more time swimming in the target quadrant on the fourth probe trial , but there were no statistically significant differences between the vehicle-treated and the SB-271046-treated groups on any of the probe trials, $F's(1,38)<1.75$, $p>0.20$.

DISCUSSION

The results of the present experiments demonstrate, for the first time, that there are differences in the pharmacological properties of the mouse 5-HT6 receptor, relative to the human and rat receptor. Although the mouse, rat and human receptors are fairly homologous (>84% identical), four critical residues had been identified for ligand binding to the 5-HT6 receptor, and one of these four residues is different in the mouse than in the rat and human receptor (Boess et al., 1998; Kohen et al., 2001). Site mutations of the rat receptor that alter the one residue that is unique to the mouse receptor, significantly affect ligand binding (Boess et al., 1998), but those site mutations were not identical to the mouse receptor sequence, and receptor binding had not been reported previously with the mouse 5-HT6 receptor. Receptor binding studies included in the present report show that both Ro 04-6790 and SB 271046 have high affinity to rat and human receptors, but Ro 04-6790 did not bind to the mouse receptor, and SB 271046 had a lower affinity at the mouse receptor than at the rat or human receptor. These results make it clear that mice should not be used to assess the therapeutic potential of 5-HT6 receptor antagonists unless the compounds are shown to bind to the mouse receptor, and the results of previous studies assessing compounds such as Ro 04-6790 in mice need to be re-evaluated (Bourson et al., 1998).

Based on the results of the receptor binding experiments, all behavioral assessments included in the present report were conducted in rats. Our results replicated the finding that 5-HT6 receptor antagonists produce stretching, a behavioral syndrome mediated by cholinergic facilitation (Bourson et al., 1995; Sleight et al., 1996; Sleight et al., 1998; Bentley et al., 1999). We detected stretching with both Ro 04-6790 and with SB-271046. Previous studies had not seen increased stretching with SB-271046 except to accentuate the effects produced by the AChE inhibitor physostigmine [(Stretton J, unpublished data, c.f. (Reavill and Rogers, 2001) and (Routledge, abstract in British Journal of Pharmacology 127(Suppl.), 21P. 1999)]. Our tests with SB-271046 may have been more sensitive to stretching behavior because we habituated our rats to the testing procedure on numerous occasions before conducting the testing. These

habituation periods reduce activity levels during the test, which make it easier to observe stretching if it occurs.

In contrast to the stretching behavior, which is consistent with facilitation of cholinergic neurotransmission, none of our efforts to assess the therapeutic potential of 5-HT6 receptor antagonists on measures of cognitive function detected any positive effects. For example, the results of our experiment with Ro 04-6790 in the conditioned fear task were not consistent with the positive effects reported with analogs of Ro 04-6790 in the passive avoidance task (Bos et al., 2001). In another experiment, we were unable to detect significant, positive effects with SB-271046 in the conditioned fear test. Even after closely replicating the methods used previously with Ro 04-6790 and SB-271046 in an autoshaping task and in the Morris water maze (Woolley et al., 2001; Rogers and Hagan, 2001; Meneses, 2001), we failed to replicate any of the positive results reported in those studies. For example, we did not see evidence of improved acquisition or retention with Ro 04-6790 in the autoshaping task. Meneses reported bar-pressing rates of 10% for the vehicle-treated control group on the test day, and in our experiments, vehicle-treated controls pressed the bar on 6-7% of the 20 trials, which is within the expected range of Meneses' experiments, but it is so low that there are potential floor effects which may reduce the sensitivity of this test. However, even with repeated testing, no significant effects were detected for Ro 04-6790 in this task, either in normal rats or in rats with scopolamine-induced deficits. In addition, even if increased bar-pressing rates had been detected in this task, additional studies would need to be conducted to rule out potential non-specific effects such as increased activity levels and/or disinhibition or impulsivity.

Likewise, we saw no evidence of increased retention with Ro 04-6790 in the Morris water maze using the same procedures reported previously (Woolley et al., 2001). The animals reached asymptotic levels during acquisition, they showed evidence of spatial mapping during retention trials, and performance declined with repeated probe trials, but there was no evidence that Ro 04-6790 improved acquisition or retention. If anything, Ro 04-6790 actually impaired performance during acquisition trials. It is not clear what the critical difference is between our experiments and the previous experiment that reported positive

results. However, it is interesting to note that in the previous Morris water maze experiment, the group treated with Ro 04-6790 performed significantly better than the vehicle-treated controls during acquisition trials, but this difference seemed to be due to the fact that the vehicle-treated controls suddenly performed worse than expected during the last 3 acquisition trials. Whatever the cause, if the difference in performance between the vehicle-treated controls and the group dosed with Ro 04-6790 is due to uncharacteristically poor performance among the vehicle-treated controls, this should not be interpreted as evidence that the drug improved performance.

Finally, we were also unable to replicate the positive effects reported for SB-271046 in the Morris water maze, although we replicated as precisely as possible the methods used previously (Rogers and Hagan, 2001). Our results suggest that cognition enhancing effects of Ro 04-6790 and SB-271046 are not reliable. In addition, we agree with previous criticisms suggesting that the effects previously reported were not necessarily evidence of therapeutic potential (Russell and Dias, 2002). For example, the effect in both previous Morris water maze studies may have been attributed to perseveration, rather than due to increased retention (Russell and Dias, 2002). In other words, even if we had replicated the results of the previous studies showing prolonged searching for the previous target location, it would not be appropriate to conclude that this effect was evidence of improved cognitive function, it could also be attributed to perseveration or impaired cognitive function, and additional experiments would have to be run to rule out that possibility.

Consistent with the results of the present experiments, other investigators have also reported difficulty in replicating the positive effects of Ro 04-6790 and SB-271046 in the Morris water maze (Russell and Dias, 2002), and in other models of cognitive function. Chronic ICV administration of antisense oligonucleotides to the 5-HT6 receptor did not affect performance in a conditioned fear task (Yoshioka et al., 1998), and 5-HT6 receptor knockouts had no effect in a novel object recognition test (reported inMartin et al., 1998). Not only are the therapeutic effects of 5-HT6 receptor antagonists in preclinical models of cognitive function in question, but studies that support the therapeutic potential of 5-HT6 receptor antagonists for cognitive deficits is complicated by at least one report that fails to support it. For example, while some

studies have suggested that 5-HT6 receptor antagonists enhance cholinergic and glutamatergic transmission, one study failed to detect an increase in hippocampal extracellular acetylcholine levels after administration of Ro 04-6790 (Shirazi-Southall et al., 2002), and another study failed to detect increases in glutamate release from frontal cortex after systemic or direct application of SB-271046 (Russell and Dias, 2002). The fact that hippocampal extracellular acetylcholine levels were elevated after administration of clozapine but not Ro 04-6790 (Shirazi-Southall et al., 2002) also suggests that atypical anti-psychotics might not enhance cognitive function in patients with schizophrenia through their action on 5-HT6 receptors. Recent studies also suggest that there are no differences in 5-HT6 receptor binding or receptor densities in schizophrenic patients (East et al., 2002), and find neither an association between 5-HT6 receptor polymorphisms and susceptibility to schizophrenia (Shinkai et al., 1999; Ohmori et al., 2001), nor an association between 5-HT6 receptor polymorphisms and response to clozapine in schizophrenic patients (Masellis et al., 2001). Another study reported that there were no significant differences in genotypic or allelic distribution of 5-HT6 receptors among AD patients and controls, which suggests that these polymorphisms probably do not represent major genetic risk factors for Alzheimer's disease (Thome et al., 2001; Orlacchio et al., 2002).

Several previous studies have reported linear dose-response curves with more and more robust, positive effects of Ro 04-6790 up to the highest dose tested, 30 mg/kg (Sleight et al., 1998; Bentley et al., 1999; Woolley et al., 2001). Ro 04-6790 also attenuated scopolamine-induced rotations with a maximal effect at 30 mg/kg (Bourson et al., 1998), therefore, we tested Ro 04-6790 at this optimal dose of 30 mg/kg during observations for stretching, in the conditioned fear task, and in the Morris water maze. Likewise, several studies have reported that consistent, robust effects were obtained with SB-271046 at 10 mg/kg (Routledge et al., 2000; Dawson et al., 2000; Dawson et al., 2001), including tests for retention in the Morris water maze (Rogers and Hagan, 2001), so we tested SB-271046 at this optimal dose in the Morris water maze. Large sample sizes were tested with these optimal doses, and when we were unable to detect significant effects with Sprague-Dawley rats in the Morris water maze, we repeated the study with Long-Evans rats. The autoshaping task was also conducted with a very large sample size and testing was continued until we could be sure that the lack of significance was not due to a floor effect. All these

decisions were made in an attempt to maximize the probability of either detecting a beneficial effect on cognitive function, or of replicating the results of previously published studies that reported statistically significant effects with 5-HT6 receptor antagonists.

Despite our best efforts to detect significant therapeutic effects on measures of cognitive function, it is impossible to prove that a treatment is inactive or that it has no therapeutic efficacy, and no matter how many studies are conducted, it is always possible that additional studies might still uncover some potential efficacy. For example, it is possible that the use of different doses or different dosing times, or the use of an experimental design that was not confounded with extinction effects, may have detected therapeutic effects of 5-HT6 receptor antagonists on measures of cognitive function. We would emphasize that our efforts to demonstrate efficacy and/or to replicate previous positive results were fairly extensive, and that we made every effort to maximize our chances of detecting therapeutic effects. However, we do not conclude from the present experiments that 5-HT6 receptor antagonists have no therapeutic efficacy. Instead, the negative results of the present experiments simply raise questions about the reliability and validity of the therapeutic potential of 5-HT6 receptor antagonists. For results to be accepted as valid, they must be reliable, and we would simply argue that the therapeutic potential of 5-HT6 receptor antagonists cannot be accepted as valid until they can be shown to produce therapeutic effects reliably.

The results of the present experiments suggest that 5-HT6 receptor antagonists may not have therapeutic potential for cognitive disorders. Alternatively, there may be some differences between the studies, which are critical for producing and detecting positive and potentially therapeutic effects. It is clearly impossible to prove that a treatment has no therapeutic potential, and replicating the procedures used in previous reports does not constitute an exhaustive assessment of the therapeutic potential of this target. However, knowledge about the reliability and robustness of the results in preclinical studies would help to more accurately assess the therapeutic potential of novel compounds and the predictive validity of the models. If a treatment such as 5-HT6 receptor antagonism ultimately does or does not have efficacy in the clinic,

determining the differences between the preclinical studies which produced positive and negative results may allow us to determine which tests and approaches appear to have greater predictive validity.

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Figure Legends

Fig. 1: Ro 04-6790, SB-271046, and clozapine inhibit ^{3}[H]LSD binding to HEK 293 cell membranes expressing the human, rat or mouse receptors.

Fig. 2: Yawning/stretching/chewing from 30-90 minutes after vehicle, physostigmine (0.1 mg/kg) or SB-271046 (30 mg/kg), n's=10-11.

Fig. 3: Yawning/stretching/chewing from 30-90 minutes after vehicle, physostigmine (0.1 mg/kg) or Ro 04-6790 (30 mg/kg), n's=8.

Fig. 4: Percent of time spent freezing in 7-minute conditioned fear test. SCOP = scopolamine; No-Shock Vehicle Controls = control rats not given any shock during conditioning session. [A] Ro 04-6790 (30 mg/kg) did not attenuate scopolamine-induced deficits, n's=19-20. [B] SB-271046 did not attenuate scopolamine-induced deficits at any dose (1.0, 10.0 and 30.0 mg/kg), n's=20-24.

Fig. 5: Percent of bar-presses in the autoshaping task on the test day. All drugs were administered immediately after the conditioning trial, 24 hours before the test trial. [A] Treatment groups were: 0 = vehicle (n=13), or 1.0, 5.0 or 10.0 mg/kg Ro 04-6790 (n's=21-22). [B] Ro 04-6790 (5.0 mg/kg) combined with vehicle or with scopolamine (0.17 mg/kg), scopolamine alone, or vehicle alone (n's=12).

Fig. 6: Percent of bar-presses in the autoshaping task with repeated test sessions. All drugs were administered after each test session. Scopolamine (0.17 mg/kg) significantly reduced acquisition of bar-pressing, but Ro 04-6790 (5 mg/kg) only produced a non-significant trend in the direction of reduced bar-pressing (n's=12).

Fig. 7: Swim latencies during acquisition training in the Morris water maze with vehicle-treated or Ro 04-6790 (30 mg/kg), n's=14-15. Asterisks indicate trials where Ro 04-6790-treated rats performed significantly different from vehicle-treated controls. [A] Albino Sprague-Dawley rats. [B] Hooded Long-Evans rats.

Fig. 8: Swim duration in target annulus and equivalent annulus areas in other quadrants during probe trials with vehicle or Ro 04-6790 (30 mg/kg), n's=14-15. Probe trials were conducted 7, 10 or 14 days after the end of acquisition training. Asterisks indicate significant differences between duration in the target annulus and each of the other quadrants, as determined by planned contrasts, p's $\leq$ 0.05. [A] albino Sprague-Dawley rats. [B] hooded Long-Evans rats.

Fig. 9: Morris water maze testing with vehicle or SB-271046 (10 mg/kg), n's=20. [A] Swim latencies during acquisition training. [B] Percent of time spent swimming in the target quadrant during probe trials immediately after the end of acquisition training (0), or 4, 7 or 10 days after the end of acquisition training.

Table 1: Potency of compounds binding to 5-HT6 receptors (nM).

Competitor	Human 5-HT6	Mouse 5-HT6	Rat 5-HT6
SB-271046	0.13	0.81	0.19
Ro 04-6790	30.50	>1000	23.07
Clozapine	6.37	3.79	5.96
Methiothepin	0.37	0.19	0.41
Serotonin	135.95	277.79	ND

Data is expressed as K_i, based on an ^{3}[H]LSD concentration of 2 nM and a K_d of 1.9 nM (Boess et al. 1997). ND is not determined.

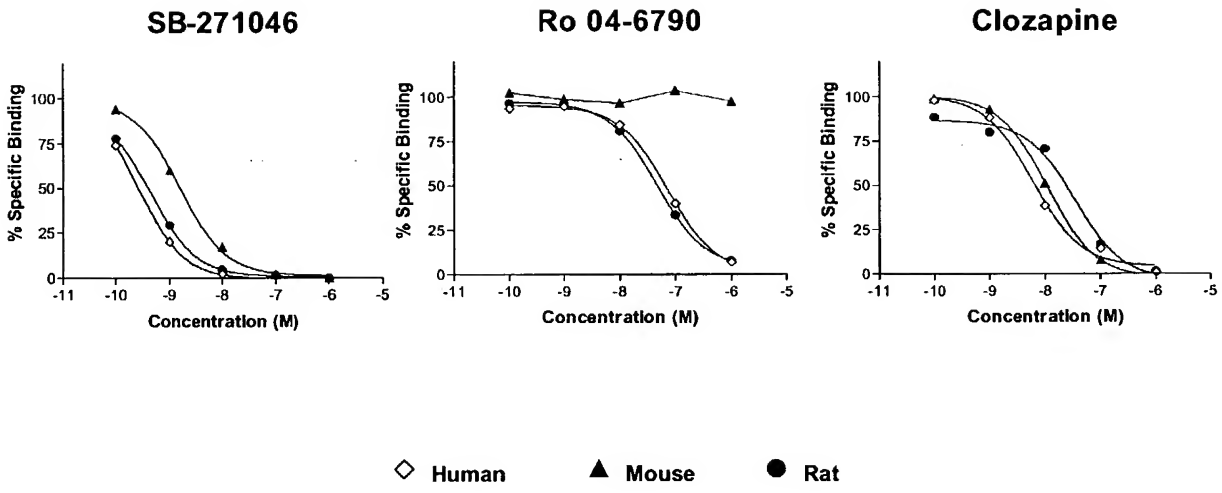
SB-271046
Ro 04-6790
Clozapine
% Specific Binding
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
Concentration (M)
Human
Mouse
Rat

FIGURE 1

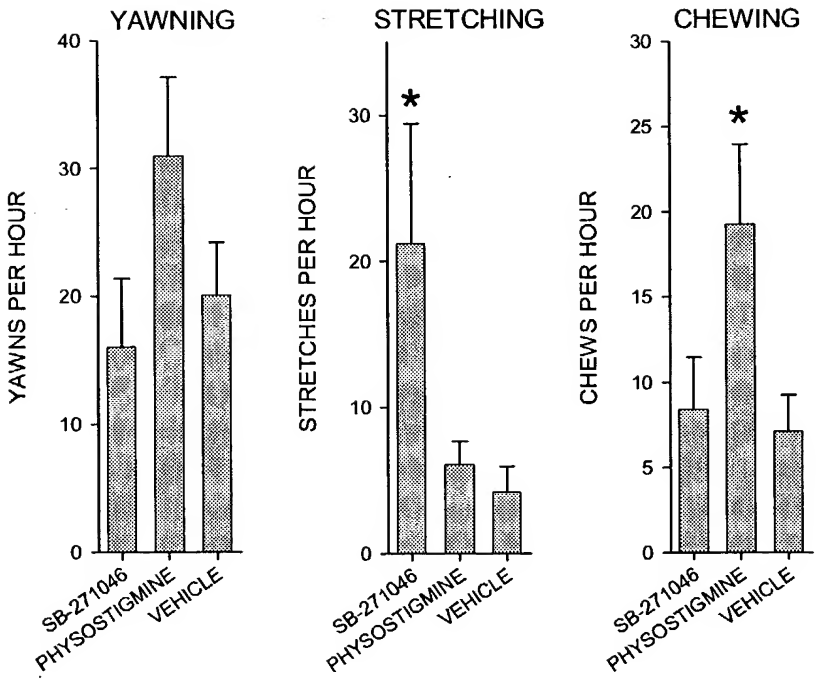
YAWNING
YAWNS PER HOUR
40
30
20
10
0
SB-271046
PHYSOSTIGMINE
VEHICLE
STRETCHING
STRETCHES PER HOUR
30
20
10
0
*
SB-271046
PHYSOSTIGMINE
VEHICLE
CHEWING
CHEWS PER HOUR
30
25
20
15
10
5
0
*
SB-271046
PHYSOSTIGMINE
VEHICLE

FIGURE 2

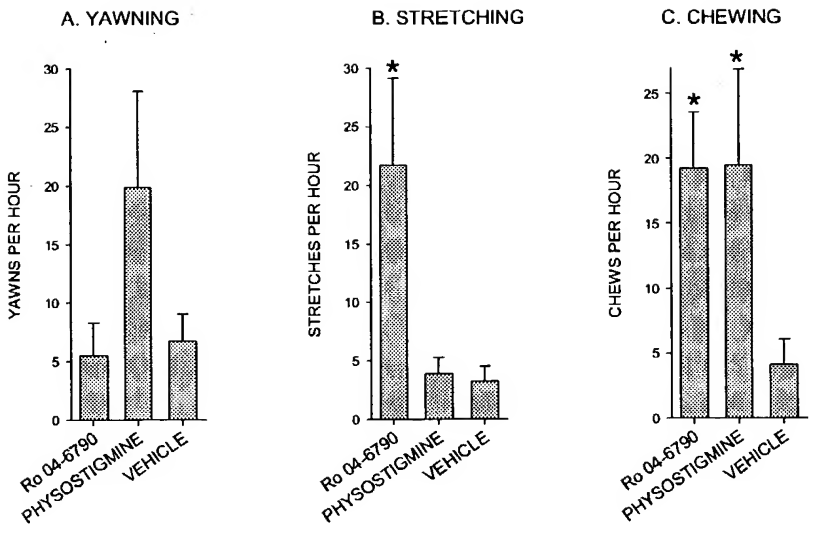
A. YAWNING
YAWNS PER HOUR
30
25
20
15
10
5
0
Ro 04-6790
PHYSOSTIGMINE
VEHICLE
B. STRETCHING
STRETCHES PER HOUR
*
30
25
20
15
10
5
0
Ro 04-6790
PHYSOSTIGMINE
VEHICLE
C. CHEWING
CHEWS PER HOUR
*
*
25
20
15
10
5
0
Ro 04-6790
PHYSOSTIGMINE
VEHICLE

FIGURE 3

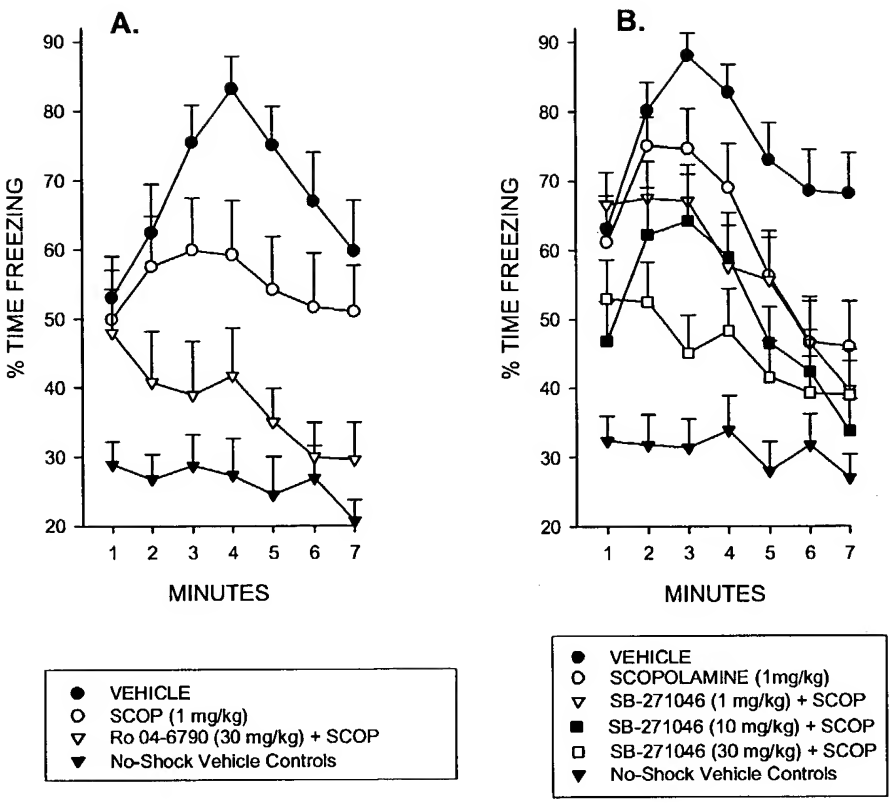
A.
% TIME FREEZING
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7
MINUTES
VEHICLE
SCOP (1 mg/kg)
Ro 04-6790 (30 mg/kg) + SCOP
No-Shock Vehicle Controls
B.
% TIME FREEZING
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7
MINUTES
VEHICLE
SCOPOLAMINE (1mg/kg)
SB-271046 (1 mg/kg) + SCOP
SB-271046 (10 mg/kg) + SCOP
SB-271046 (30 mg/kg) + SCOP
No-Shock Vehicle Controls

FIGURE 4

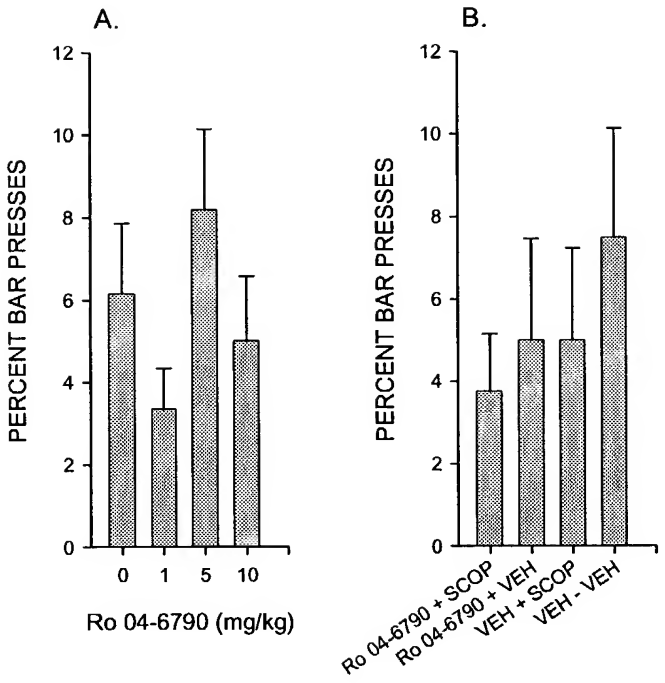
A.
B.
PERCENT BAR PRESSES
12
10
8
6
4
2
0
0
1
5
10
Ro 04-6790 (mg/kg)
Ro 04-6790 + SCOP
Ro 04-6790 + VEH
VEH + SCOP
VEH - VEH

FIGURE 5

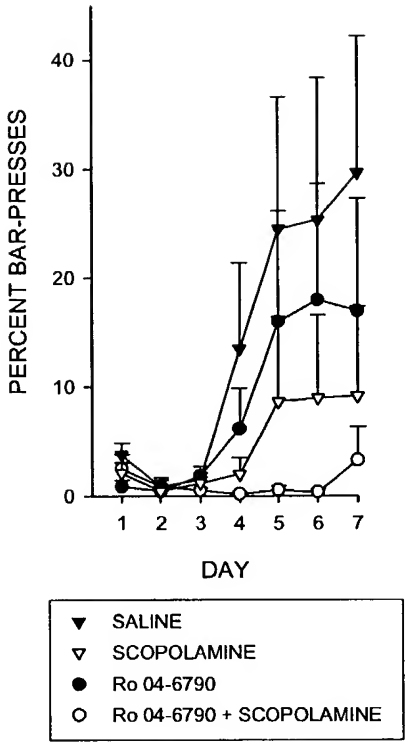
PERCENT BAR-PRESSES
40
30
20
10
0
1
2
3
4
5
6
7
DAY
SALINE
SCOPOLAMINE
Ro 04-6790
Ro 04-6790 + SCOPOLAMINE

FIGURE 6

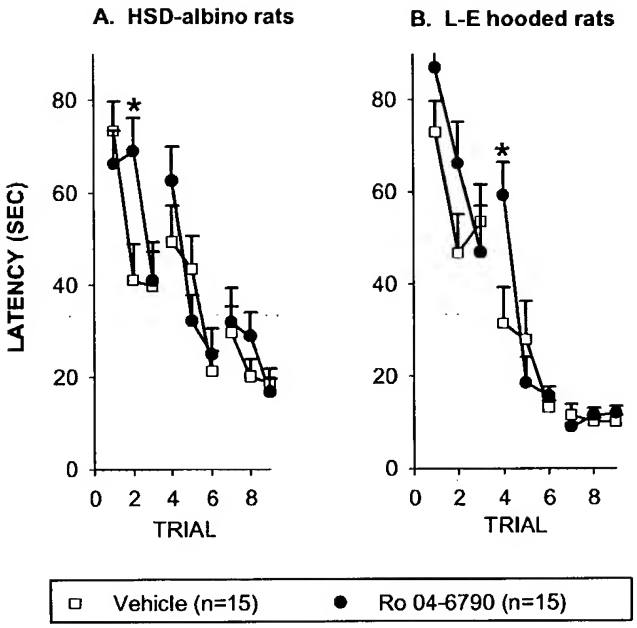
A. HSD-albino rats
B. L-E hooded rats
LATENCY (SEC)
80
60
40
20
0
0 2 4 6 8
TRIAL
*
Vehicle (n=15)
Ro 04-6790 (n=15)

FIGURE 7

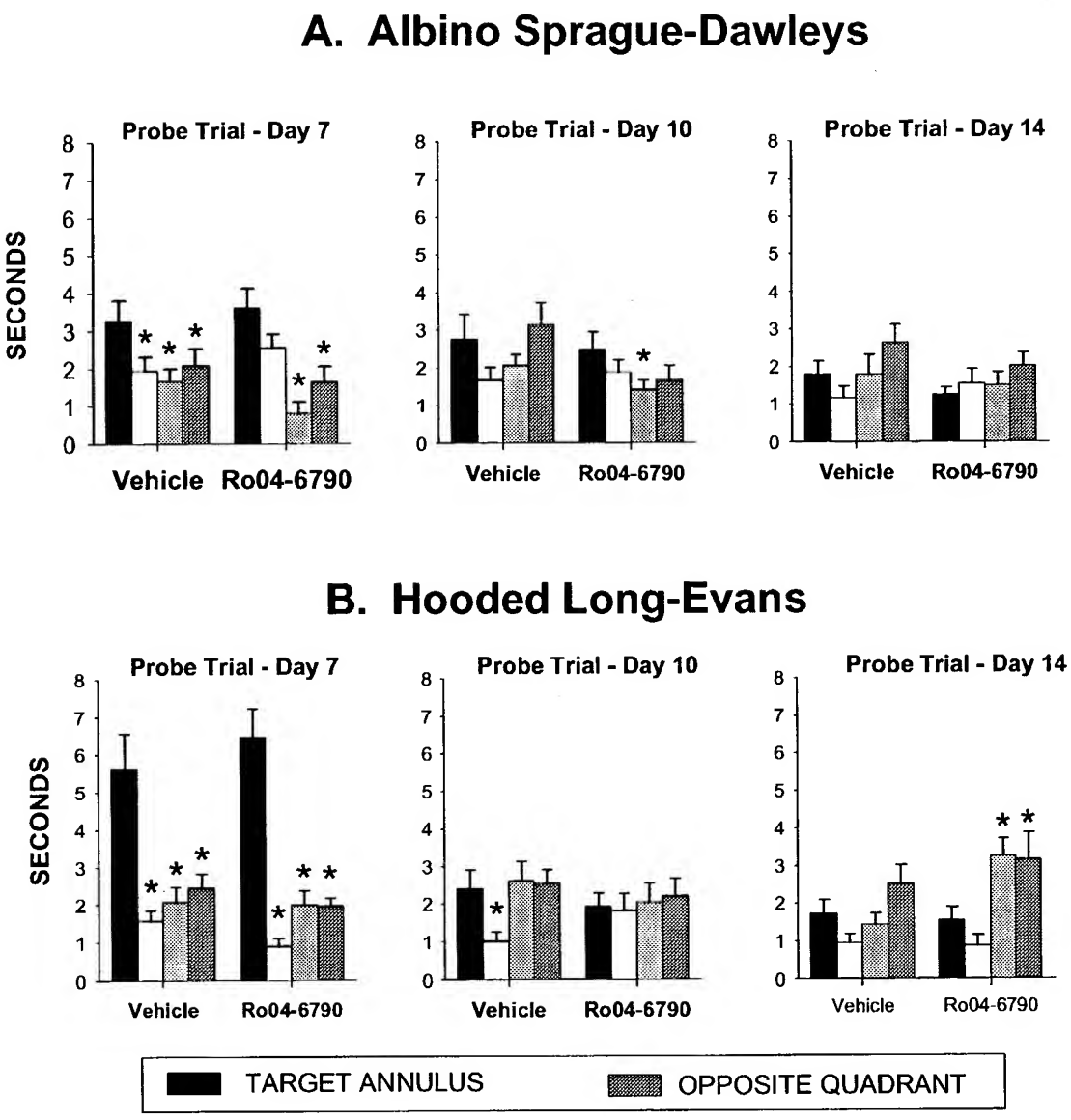
A. Albino Sprague-Dawleys
Probe Trial - Day 7
Probe Trial - Day 10
Probe Trial - Day 14
SECONDS
Vehicle
Ro04-6790
B. Hooded Long-Evans
TARGET ANNULUS
OPPOSITE QUADRANT

FIGURE 8

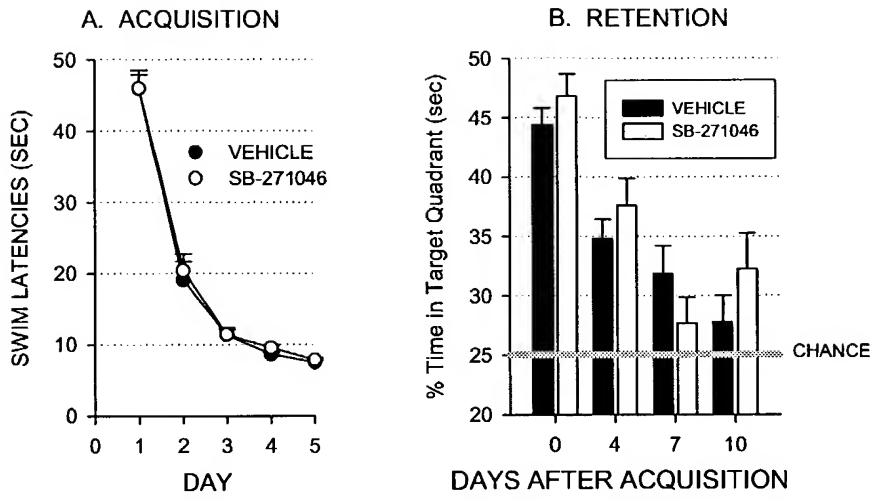
A. ACQUISITION
SWIM LATENCIES (SEC)
50
40
30
20
10
0
0 1 2 3 4 5
DAY
VEHICLE
SB-271046
B. RETENTION
% Time in Target Quadrant (sec)
50
45
40
35
30
25
20
0 4 7 10
DAYS AFTER ACQUISITION
VEHICLE
SB-271046
CHANCE

FIGURE 9

0026-895X/97/030515-09$3.00/0

MOLECULAR PHARMACOLOGY, 52:515–523 (1997).

Interaction of Tryptamine and Ergoline Compounds with Threonine 196 in the Ligand Binding Site of the 5-Hydroxytryptamine$_6$ Receptor

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SUMMARY

We examined the ligand-binding site of the 5-hydroxytryptamine$_6$ (5-HT$_6$) receptor using site-directed mutagenesis. Interactions with residues in two characteristic positions of transmembrane region V are important for ligand binding in several bioamine receptors. In the 5-HT$_6$ receptor, one of these residues is a threonine (Thr196), whereas in most other mammalian 5-HT receptors, the corresponding residue is alanine. After transient expression in human embryonic kidney 293 cells, we determined the effects of the mutation T196A on [^{3}H]*d*-lysergic acid diethylamide (LSD) binding and adenylyl cyclase stimulation. This mutation produced a receptor with a 10-fold reduced affinity for [^{3}H]LSD and a 6-fold reduced affinity for 5-HT. The potency of both LSD and 5-HT for stimulation of adenylyl cyclase was also reduced by 18- and 7-fold, respectively. The affinity of other N1-unsubstituted ergolines (e.g., ergotamine, lisuride) was reduced 10–30-fold, whereas the affinity of N1-methylated ergolines (e.g., metergoline, methysergide, mesulergine) and other ligands, such as methiothepine, clozapine, ritanserin, amitriptyline, and mianserin, changed very little or increased. This indicates that in wild-type 5-HT$_6$ receptor, Thr196 interacts with the N1 of N1-unsubstituted ergolines and tryptamines, probably forming a hydrogen bond. Based on molecular modeling, a serine residue in transmembrane region IV of the 5-HT$_{2A}$ receptor has previously been proposed to interact with the N1-position of 5-HT. When the corresponding residue of the 5-HT$_6$ receptor (Ala154) was converted to serine, no change in the affinity of twelve 5-HT$_6$ receptor ligands or in the potency of 5-HT and LSD could be detected, suggesting that this position does not contribute to the ligand binding site of the 5-HT$_6$ receptor.

To date, 14 distinct mammalian 5-HT (serotonin) receptors have been identified. The known 5-HT receptors include a ligand-gated ion channel (5-HT$_3$ receptor) and 13 G protein-coupled receptors (1, 2). Unlike the classic 5-HT receptors, the 5-HT$_6$ receptor was first discovered by cloning from rat striatal cDNA but had not been previously identified as a pharmacological entity in physiological or radioligand binding experiments (3, 4). Subsequently, the human 5-HT$_6$ receptor was isolated by homology screening (5). The highest levels of 5-HT$_6$ receptor mRNA are present in olfactory tubercle, nucleus accumbens, striatum, and hippocampus (3, 4, 6, 7). In addition to these regions, 5-HT$_6$ receptor-like immunoreactivity was identified in frontal and entorhinal cortex and the molecular layer of the cerebellum (8). The functional significance of this receptor has been investigated using intracerebroventricular injection of 5-HT$_6$ receptor-specific antisense oligonucleotides, which produced a behavioral syndrome, suggesting effects on dopaminergic and/or cholinergic neurotransmission (9, 10).

The 5-HT$_6$ receptor displays a characteristic pharmacological profile, as was shown in competition studies with either ^{125}I-LSD or [^{3}H]5-HT, both of which bind to the 5-HT$_6$ receptor with high affinity (3). Many nonselective compounds, such as tricyclic antidepressant drugs and a large number of antipsychotic agents, tryptamine, and ergoline derivatives, interact with the 5-HT$_6$ receptor (3, 11, 12). Because no selective ligands are available, identification of functional 5-HT$_6$ receptors in physiological preparations can be only tentative. A model of the 5-HT$_6$ receptor binding site may suggest modifications of known ligands to improve the affinity and selectivity for this receptor. To verify and adapt models that have been proposed for other G protein-coupled receptors, we attempted to identify residues contributing to the ligand binding site of the 5-HT$_6$ receptor through the use of site-directed mutagenesis.

There is general agreement that for this class of bioamine receptors, the ligand binding site is formed by seven transmembrane helices present in all members of the family (13–15). In these transmembrane regions, the 5-HT$_6$ receptor shows highest homology to other 5-HT receptors (36–41%

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; LSD, *d*-lysergic acid diethylamide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

sequence identity), but the homology to adrenergic, dopamine, and histamine receptors is also quite high (30–35% identity depending on which segments are included in the comparison). In site-directed mutagenesis and modeling studies with other bioamine receptors, several specific regions were consistently found to be involved in ligand binding and receptor activation. In transmembrane helix V of the hamster β_2-adrenergic receptor, replacement of either of two serine residues attenuated the activity of catecholamine agonists at the receptor (16) (Fig. 1). Removal of the catechol hydroxyl moieties from the aromatic ring of the ligand mimicked the effects of the mutation, suggesting that in wild-type receptors, hydrogen bond interactions are formed between the serine residues and the *meta*- and *para*-hydroxyl group of agonists. These two or three characteristically spaced serine residues (one helix turn or three or four residues apart) were subsequently shown to contribute to agonist binding in α_{2A}- and α_1-adrenergic receptors (17, 18) and both D_1 and D_2 dopamine receptors (19–22). In the histamine receptors, these positions are occupied by threonine and asparagine (H_1 receptor) or aspartate and threonine (H_2), and mutations of these residues also affect ligand binding (23–26). In all G protein-coupled 5-HT receptors cloned to date, the first of these "hydrogen bonding" positions is occupied by a serine or threonine residue and frequently preceded by another serine (see Fig. 1). In the 5-HT_{1A} receptor, a serine and a threonine residue are next to each other at this position. Replacement of either residue by alanine reduced the affinity of 5-HT and its ability to stimulate GTPase activity, suggesting the disruption of a normally present hydrogen bond with the hydroxyl-group on the indole ring of 5-HT (27). The second potential hydrogen bonding position in transmembrane region V is occupied by an alanine in 11 of the 13 cloned mammalian G protein-coupled 5-HT receptor subtypes. The only exceptions are the rat and human 5-HT_6 receptors (threonine) and the human, pig, and squirrel monkey 5-HT_{2A} receptors (serine), whereas the rat 5-HT_{2A} receptor has an alanine residue in this position (like all other subtypes). This single amino acid variation is responsible for dramatic species differences in the affinity of N1-substituted or unsubstituted ergolines and tryptamines for rat compared with human, pig, or squirrel monkey 5-HT_{2A} receptors, as was demonstrated by reversal of the pharmacology by point mutations in both the human and rat receptor (28–32). This position contributes to the subtype selectivity of ligands because replacement of the wild-type alanine of the human 5-HT_{2C} receptor by serine changes the affinity of several ergoline compounds to values closer to their affinity for the human 5-HT_{2A} receptor, and vice versa (33).

To determine whether this second potential hydrogen bonding position available in the 5-HT_6 receptor contributes to ligand binding, we replaced the threonine residue present at the corresponding position of the rat 5-HT_6 receptor (Thr196) with alanine (T196A), the residue present in most other mammalian 5-HT receptors.

Using a G protein-coupled receptor model based on the structure of bacteriorhodopsin, Hibert *et al.* (13) suggested a number of potential interactions of 5-HT with the 5-HT_{2A} receptor, including a hydrogen bond between the 5-hydroxyl group of 5-HT and a serine in transmembrane V (in the first hydrogen bonding position discussed above) and a second hydrogen bond between a serine residue in transmembrane IV and the indole-nitrogen (N1) of 5-HT (13). This serine residue is conserved in most cloned mammalian 5-HT receptors with the exception of the 5-HT_{1A} receptors (glycine), 5-HT_4 receptor (proline), and 5-HT_6 receptors (alanine) (Fig. 2). We tested whether the substitution of Ala154 by serine (A154S) (i.e., the introduction of a hydroxyl group) would allow the formation of a new hydrogen bond and thus change the affinity or agonist activity of 5-HT_6 receptor ligands.

rat 5-HT_{1A}	YTIY**ST**FGAFYIPLLLMLVL
rat 5-HT_{1B}	YTVY**ST**VGAFYLPTLLLIAL
rat 5-HT_{1D}	YTIY**ST**CGAFYIPSILLIIL
human 5-HT_{1E}	YTIY**ST**LGAFYIPLTLILIL
rat 5-HT_{1F}	STIY**ST**FGAFYIPLVLILIL
rat 5-HT_{2A}	FVLIG**S**FV**A**FFIPLTIMVIT
human 5-HT_{2A}	FVLIG**S**FV**S**FFIPLTIMVIT
rat 5-HT_{2B}	FMLFG**S**LAAFFAPLTIMIVT
rat 5-HT_{2C}	FVLIG**S**FV**A**FFIPLTIMVIT
rat 5-HT_4	YAITC**S**VVAFYIPFLLMVLA
rat 5-HT_{5A}	YTVF**ST**VGAFYLPLCVVLFV
rat 5-HT_{5B}	YAVF**ST**CGAFYVPLAVVLFV
rat 5-HT_6	FVLVA**S**GV**T**FFLPSGAICFT
rat 5-HT_7	YTIY**ST**AVAFYIPMSVMLFM
human D_1	YAIS**S**S**S**VI**S**FYIPVAIMIVT
human D_2	FVVY**S**S**S**IV**S**FYVPFIVTLLV
rat α_{1A}	YVLF**S**ALG**S**FYVPLAIILVM
human α_{2A}	YVIS**S**CIG**S**FFAPCLIMILY
hamster β_2	YAIA**S**S**S**IV**S**FYVPLVVMVFV
guinea pig H_1	FKVM**T**AII**N**FYLPTLLMLWF
dog H_2	YGLV**D**GLV**T**FYLPLLVMCIT

Fig. 1. Alignment of transmembrane region V of mammalian 5-HT and related bioamine receptors. *Bold*, conserved serine and threonine residues in the first potential hydrogen bonding position(s) of the 5-HT receptors. The role of the *outlined* residues in ligand binding has been examined in the receptors listed and/or in species homologues with the help of site-directed mutagenesis. In the current study, Thr196 of the 5-HT_6 receptor has been mutated to alanine, the residue found in most other 5-HT receptors.

Experimental Procedures

Cloning of the rat 5-HT_6 receptor and site-directed mutagenesis. A cDNA clone representing the 5-HT_6 receptor was generated from rat striatal mRNA by reverse transcription-polymerase chain reaction using primers based on the published rat 5-HT_6

rat 5-HT_{1A}	LISLTWLIGFLISIPPM
rat 5-HT_{1B}	MIVLVWVF**S**ISISLPPF
rat 5-HT_{1D}	MIAAVWAI**S**ICISIPPL
human 5-HT_{1E}	MILTVWTI**S**IFISMPPL
rat 5-HT_{1F}	TITTVWVI**S**VFISVPPL
rat 5-HT_{2A}	KIIAVWTI**S**VGISMPIP
rat 5-HT_{2B}	KITVVWLI**S**IGIAIPVP
rat 5-HT_{2C}	KIAIVWAI**S**IGVSVPIP
rat 5-HT_4	MLGGCWVIPMFISFLPI
rat 5-HT_{5A}	MILLTWAL**S**AVISLAPL
rat 5-HT_{5B}	MIAITWAL**S**ALIALAPL
rat 5-HT_6	LILGAWSL**A**ALASFLPL
rat 5-HT_7	MILSVWLL**S**ASITLPPL

Fig. 2. Alignment of part of transmembrane region IV of mammalian 5-HT receptors. *Bold*, conserved serine residues. Ala154 of the 5-HT_6 receptor (in outline view) has been mutated in the present study.

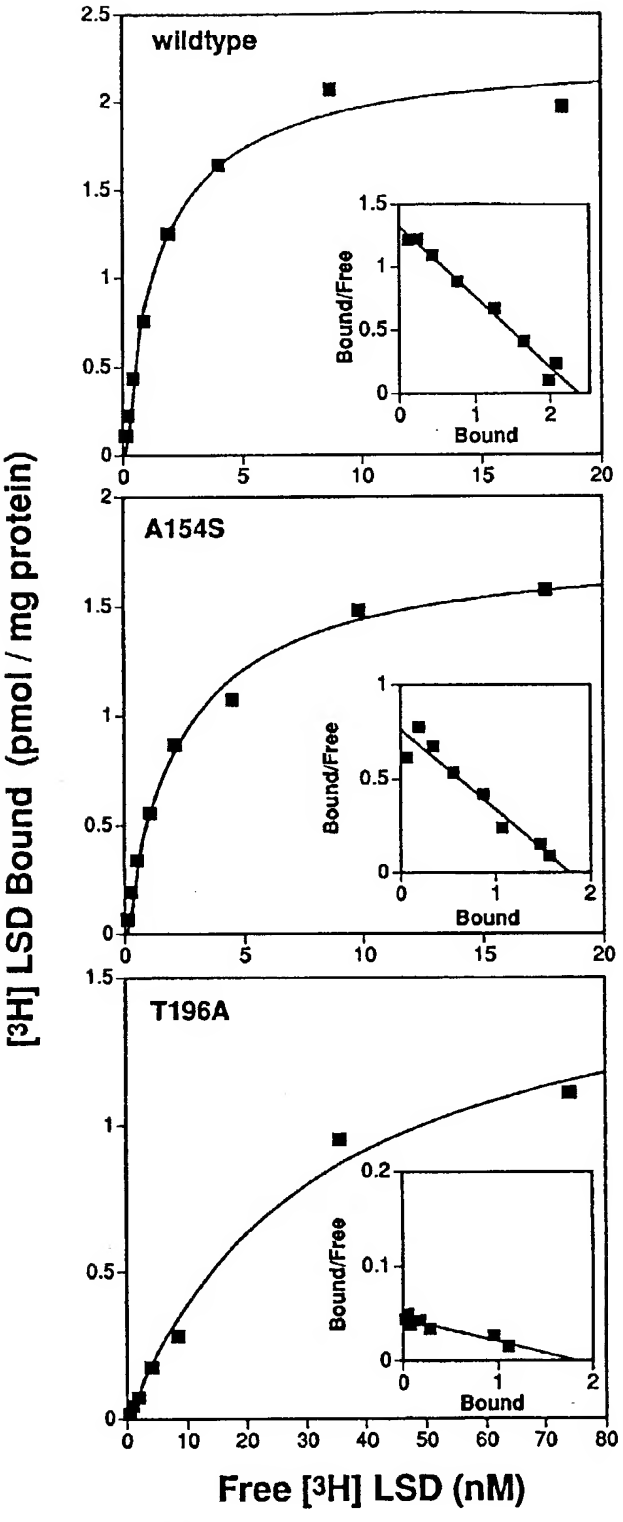

Fig. 3. [^{3}H]LSD binding to HEK 293 membranes expressing wild-type 5-HT$_6$ receptor or mutant receptors A154S or T196A. The examples shown are from HEK 293 cells transfected with the same amount of pcDNAI-5-HT$_6$ receptor wild-type or mutant constructs. *Insets*, Scatchard transformation of saturation binding data. The B_{max} *values of the experiments shown are 2.4 (wild-type), 1.8 (A154S), and 1.9 (T196A) pmol/mg of protein, and the* K_d values are 1.8 nM (wild-type), 2.3 nM (A154S), and 27.7 nM (T196A).

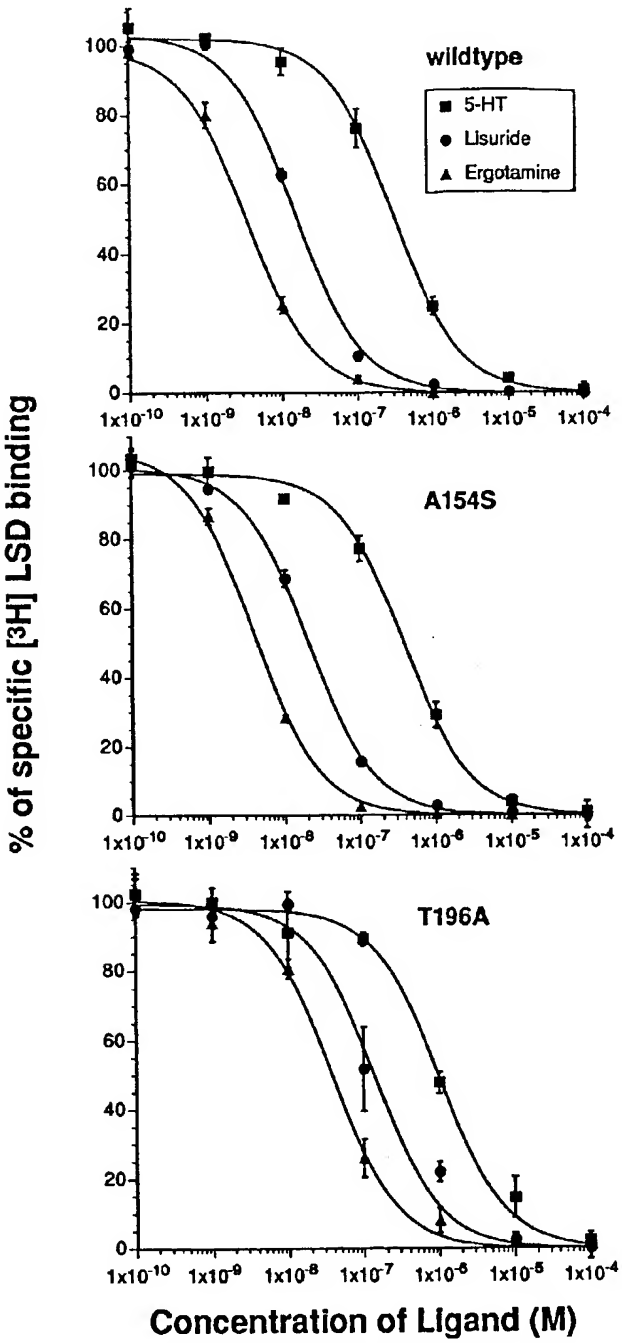

Fig. 4. Interaction of wild-type and mutant receptors with 5-HT and N1-unsubstituted ergolines. [^{3}H]LSD competition experiments were conducted for wild-type 5-HT$_6$ receptor, mutants A154S and T196A with 5-HT (■), and the N1-unsubstituted ergolines lisuride (●) and ergotamine (▲). Data are mean ± standard error from three experiments performed as described in Experimental Procedures.

sequence (3)[1] and subcloned into the plasmid pAMP1 using the CloneAmp system (Life Technologies, Eggenstein, Germany). One clone was identified that matched exactly the sequence of Kohen *et al.* (5)[2] with the exception of a single base exchange that did not alter

[1] GenBank accession number L03202.
[2] GenBank accession number L41146.

TABLE 1

Pharmacological profile of mutant 5-HT$_6$ receptors

Affinites of 5-HT, N1-unsubstituted ergolines (LSD, lisuride, ergotamine), and N1-methylated ergolines (metergoline, methysergide, mesulergine) as well as structurally unrelated ligands for wild-type 5-HT$_6$ receptor compared with mutant receptors T196A and A154S. [^{3}H]LSD binding assays were performed as described in Experimental Procedures. Values are mean ± standard error from three to five experiments. To determine whether the affinities for T196A were significantly different from the wild-type values, the individual pK_i values were compared using an unpaired *t* test.

	Wild-type 5-HT$_6$ receptor		Mutant T196A		Mutant A154S	
	K_i (or K_d)	Hill slope	K_i (or K_d)	Hill slope	K_i (or K_d)	Hill slope
	nM		nM		nM	
[^{3}H]LSD	1.9 ± 0.2	1.01 ± 0.03	33.9 ± 7.1[b]	0.98 ± 0.01	2.1 ± 0.1	1.02 ± 0.02
Lisuride	10.9 ± 1.8	1.13 ± 0.06	372.7 ± 183.7[a]	0.86 ± 0.02	13.8 ± 0.9	0.99 ± 0.12
Ergotamine	2.4 ± 0.2	1.01 ± 0.01	38.6 ± 8.3[b]	1.00 ± 0.12	3.5 ± 0.8	1.19 ± 0.05
5-HT	168.2 ± 19.5	0.94 ± 0.04	992.5 ± 284.4[b]	0.89 ± 0.13	231.1 ± 38.5	0.95 ± 0.01
Metergoline	56.7 ± 11.3	0.89 ± 0.03	60.4 ± 12.6	0.97 ± 0.22	56.3 ± 1.7	0.93 ± 0.03
Methysergide	289.8 ± 92.3	0.74 ± 0.04	41.0 ± 5.8[a]	0.98 ± 0.09	278.5 ± 29.4	0.89 ± 0.03
Mesulergine	2662.9 ± 681.0	0.90 ± 0.05	725.0 ± 93.0[a]	0.85 ± 0.11	1879.3 ± 138.6	0.89 ± 0.02
Methiothepin	6.2 ± 2.2	1.33 ± 0.12	6.6 ± 2.7	1.17 ± 0.16	4.4 ± 1.0	1.13 ± 0.19
Clozapine	16.6 ± 4.4	1.01 ± 0.07	44.1 ± 9.4	0.74 ± 0.14	16.4 ± 3.6	0.97 ± 0.08
Amitriptyline	81.3 ± 6.2	0.82 ± 0.07	64.1 ± 24.2	0.64 ± 0.04	95.5 ± 3.3	0.88 ± 0.02
Ritanserin	35.5 ± 2.8	0.98 ± 0.08	71.3 ± 27.6	0.63 ± 0.08	52.4 ± 4.4	1.06 ± 0.06
Mianserin	113.1 ± 19.6	1.02 ± 0.04	37.0 ± 11.5	0.92 ± 0.15	129.4 ± 47.6	0.91 ± 0.03

[a] significantly different ($p < 0.01$).
[b] significantly different ($p < 0.001$).

the amino acid sequence. Further details are provided in Boess *et al.* (12). An *Eco*RI/*Xba*I restriction fragment of the pAMP1–5-HT$_6$ construct was ligated into the expression vector pcDNA1.1amp (InVitrogen, San Diego, CA) and into the phagemid pAlter (Promega, Madison, WI). Mutagenesis was performed using the Altered Sites Mutagenesis kit (Promega). Thr196 was mutated to alanine (T196A) using the oligonucleotide 5′-GTCCGGCGTCGCCTTTTTCCT-3′. Ala154 was mutated to serine (A154S) using the mutagenic oligonucleotide 5′-GGTGCCTGGAGCCTCAGCGCGCTTGCCTCCTTC-3′. This oligonucleotide introduced an additional silent mutation, creating a new *Bss*H II restriction site. After transfer into the expression vector pcDNA1.1amp, mutations were confirmed by sequencing the entire coding region using an automated fluorescent sequencing system (ALF; Pharmacia, Vienna, Austria).

Transient transfection of HEK 293 cells. HEK 293 cells were maintained in DMEM plus 10% FBS containing 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (5% CO_2). HEK 293 cells (80–90% confluent in 75- or 185-cm^2 flasks) were cotransfected with the construct pcDNA1.1amp-5-HT$_6$ (8 or 20 μg) containing the coding region of wild-type or mutant 5-HT$_6$ receptor according to the Lipofectamine transfection protocol (Life Technologies). After 1 day, the medium was replaced by fresh DMEM plus 10% FBS.

Membrane preparation and receptor binding assays. Two days after transfection with the pcDNA1.1amp constructs containing mutant or wild-type 5-HT$_6$ receptor coding regions, the cells were detached with ice-cold 50 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$ and 0.5 mM EDTA. The cells were disrupted using a Polytron homogenizer (15 sec at maximal speed) at a concentration corresponding to ~2 × 10^6 cells/ml. This homogenate was centrifuged at 50,000 × *g* for 30 min, resuspended in the same volume, and centrifuged again. At this stage, the resulting pellets were either stored at −20° or used immediately in binding assays. 5-HT$_6$ receptor binding assays were performed using [^{3}H]LSD (specific activity, 82 Ci/mmol; Amersham, Little Chalfont, UK) as radioligand. Membranes were resuspended in assay buffer (50 mM Tris·HCl, 10 μM pargyline, 5 mM $MgCl_2$, 0.5 mM EDTA and 0.1% ascorbic acid, pH 7.4). Binding assays consisted of 100 μl of membranes (corresponding to 4 × 10^5 cells/assay tube), 50 μl of [^{3}H]LSD, and 50 μl of displacing drug or assay buffer (final assay volume, 200 μl). Nonspecific binding was measured in the presence of 100 μM 5-HT or 10 μM methiothepine. Saturation experiments were performed using eight concentrations of [^{3}H]LSD (final concentrations, 0.163–20 nM for wild-type and mutant A154S and 0.625–80 nM for mutant T196A). Competition assays were performed in the presence of seven concentrations of the displacing ligands (10^{-10} to 10^{-4} M) and 1 nM [^{3}H]LSD (5 nM for mutant T196A). Incubations were performed at 37° for 60 min and terminated by rapid filtration through Whatman (Maidstone, UK) GF/B filters pretreated with polyethyleneimine (0.3%). The filters were washed three times with 2 ml of Tris·HCl (50 mM; pH 7.4), and the radioactivity retained on the filters was measured by scintillation spectroscopy in 2 ml of scintillation fluid. All experiments were performed in triplicate and repeated at least three times. Values are given as mean ± standard error. Data were analyzed using the programs EBDA and LIGAND (34, 35). Protein concentrations were determined using the BCA method (Pierce Chemical, Indianapolis, IN).

Adenylyl cyclase measurements. Two days after transfection, cells grown in DMEM plus 10% FBS (dialyzed) were washed once with DMEM without phenol red (DMEM$^-$), detached with PBS plus 1 mM EDTA, and washed twice with DMEM$^-$ (470 × *g*, 5 min). The final cell density was adjusted to ~1.25 × 10^6 cells/ml. Aliquots of 80 μl were transferred to 96-well plates (ca. 10^5 cells/well) and incubated at 37° in a humidified atmosphere for 30 min. 5-HT or LSD, combined with pargyline and the phosphodiesterase inhibitor Ro 20–1724, was added in a volume of 20 μl/well (final incubation volume, 100 μl/well; final concentration of agonists, 10^{-10} to 10^{-3} M; pargyline, 20 μM; Ro 20–1724, 100 μM). After 20 min at 37° in a humidified atmosphere (5% CO_2), the incubation was terminated by the addition of 200 μl of ethanol/well. After ≥2 hr at −20°, the plates were centrifuged for 5 min at 470 × *g* (4°), and 75-μl aliquots of the supernatant were transferred to OptiPlates (Packard, Meriden, CT), evaporated under vacuum, and resuspended in 0.05 M acetate buffer. The concentration of cAMP was determined using the BIOTRAK cAMP [^{125}I] Scintillation Proximity Assay system (Amersham) adapted to 96-well plates. The concentration effect curves were analyzed using the equation $E = B + E_{max} * x/(EC_{50} + x)$, where E and E_{max} are the measured and maximum effects (cAMP/well), respectively; B is the basal cAMP level, and *x* is the concentration of agonist.

Materials. [^{3}H]LSD (specific activity, 82 Ci/mmol) was obtained from Amersham. 5-HT was from FLUKA (Buchs, Switzerland), and ergotamine was from Sigma (Buchs, Switzerland). Mesulergine, metergoline, methysergide, lisuride, methiothepine, clozapine, amitriptyline, ritanserin, mianserin, and pargyline were purchased from Research Biochemicals (Natick, MA). DMEM, FBS, penicillin, streptomycin, and geneticin were obtained from Gibco Life Technologies

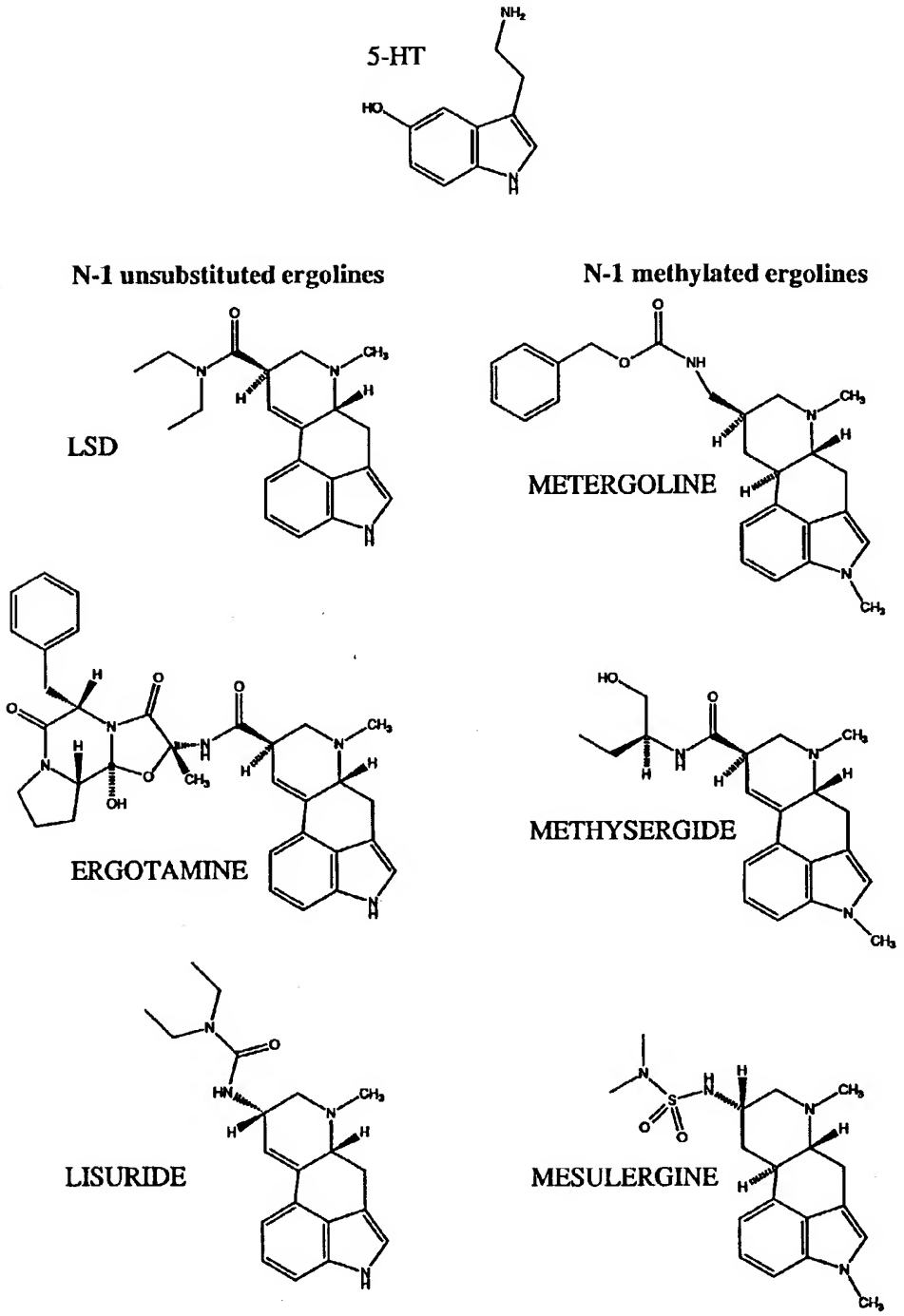

Fig. 5. Structure of 5-HT and the ergoline compounds used in the study.

(Basel, Switzerland). Ro 20–1724 and LSD were synthesized at F. Hoffmann-La Roche (Basel, Switzerland)

Results

Expression in HEK 293 cells and [^{3}H]LSD binding. Transient transfection of HEK 293 cells with wild-type 5-HT$_6$ receptor or either mutant receptor construct resulted in high expression levels of 1–3 pmol/mg of protein as determined in [^{3}H]LSD binding assays. When the transfections were performed in parallel using the same batch of HEK 293 cells, the amount of receptor expressed per mg of protein was similar for wild-type and mutant receptors (Fig. 3). The K_d values for wild-type 5-HT$_6$ receptor and mutant A154S were not significantly different (1.9 ± 0.3 and 2.1 ± 0.1 nM, respectively). However, the affinity of [^{3}H] LSD for mutant T196A was reduced by a factor of 16 (K_d = 33.9 ± 7.1 nM; $p < 0.001$).

Comparison of the pharmacological profile of wild-type and mutant receptors. In competition assays with [^{3}H] LSD, the affinity of the endogenous agonist 5-HT for the mutant receptor T196A was reduced 6-fold compared with wild-type 5-HT$_6$ receptor (Fig. 4, Table 1). The affinities of the N1-unsubstituted ergolines ergotamine and lisuride were reduced 16- and 34-fold, respectively

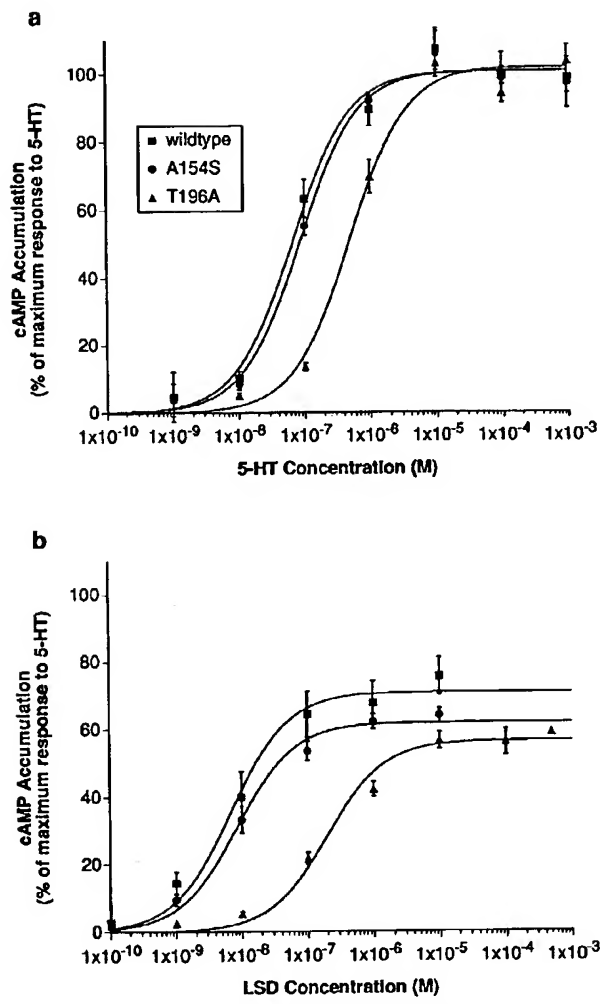

Fig. 6. Functional responses of wild-type and mutant 5-HT_6 receptors. Stimulation of cAMP accumulation in HEK 293 cells expressing wild-type 5-HT_6 receptor (■), mutant A154S (●), or mutant T196A (▲) by 5-HT (a) and LSD (b). Values are expressed as percentage of maximum cAMP accumulation obtained with 5-HT at the wild-type 5-HT_6 receptor. Data are the mean ± standard error of three experiments performed as described in Experimental Procedures.

(Fig. 4, Table 1). In contrast, the affinity of N1-methylated ergolines for the mutant T196A did not change (metergoline) or increased 4–7-fold (methysergide, mesulergine) (Table 1) (see Fig. 5 for structures). The affinity of several other high affinity 5-HT_6 receptor ligands that are neither tryptamine nor ergoline derivatives (methiothepine, clozapine, amitriptyline, ritanserin, and mianserin) did not change significantly (Table 1). The Hill slopes of the ligands tested were not significantly different between wild-type and T196A mutant 5-HT_6 receptors, with the exception of lisuride and ritanserin, which had lower Hill slopes at the mutant receptor ($p < 0.05$, unpaired t test). Neither of the compounds examined showed significant changes in affinity for the second mutant (A154S) (Fig. 4; Table 1).

Comparison of the functional properties of wild-type and mutant 5-HT_6 receptors. The basal levels of cAMP accumulation (after 20 min of incubation without agonist) were 1.3 ± 0.3, 0.9 ± 0.2, and 0.9 ± 0.2 pmol/10^5 cells for HEK 293 cells expressing wild-type, mutant A154S, and mutant T196A 5-HT_6 receptors, respectively. HEK 293 cells expressing the wild-type 5-HT_6 receptor or either of the mutant receptors responded to 5-HT and LSD with increased cAMP production (Fig. 6), whereas nontransfected cells did not respond in the concentration range tested. The maximum level of cAMP accumulation obtained after stimulation with 5-HT was 27.5 ± 6.9 pmol/10^5 cells for wild-type 5-HT_6 receptor, 25.1 ± 4.7 pmol/10^5 cells for mutant A154S, and 21.7 ± 3.6 pmol/10^5 cells for mutant T196A (mean ± standard error, three experiments). Therefore, the mutations did not influence the efficiency of receptor/G protein interactions. The maximum levels of cAMP accumulation obtained with LSD were 67 ± 9% (wild-type), 66 ± 7% (A154S), and 69 ± 12% (T196A) of the maximum observed with 5-HT. This indicates that LSD is a partial agonist at the 5-HT_6 receptor and the changes introduced in the mutants do not alter the partial agonist character of this ligand. In cells expressing wild-type 5-HT_6 receptor, 5-HT stimulated cAMP accumulation with an EC_{50} value of 74 ± 22 nM. LSD was more potent, with an EC_{50} of 16 ± 7 nM. Replacement of Ala154 by a serine residue in transmembrane region IV (mutant A154S) did not significantly change the potency of either 5-HT (EC_{50} = 89 ± 13 nM) or LSD (EC_{50} = 17 ± 8 nM). However, in cells expressing the mutant T196A, the agonist potency of 5-HT was reduced 7-fold (EC_{50} = 540 ± 73 nM, $n = 3, p < 0.01$), and that of LSD was reduced 18-fold (EC_{50} = 290 ± 74 nM, $p < 0.05$) (Fig. 6).

Change in free energy. For LSD and 5-HT, the change in binding energy Δ(ΔG) introduced by the mutation T196A could be calculated both from the EC_{50} values determined in adenylyl cyclase stimulation assays and the K_d and K_i values determined in [^{3}H]LSD binding experiments (Table 2). The values calculated for 5-HT (5.1 and 4.6 kJ/mol) were lower than those observed for LSD (7.5 and 7.3 kJ/mol). The change in free energy observed for ergotamine (7.2 kJ/mol) and lisuride (9.1 kJ/mol) calculated from the K_i values was also higher than for 5-HT.

Discussion

The 5-HT_6 receptor has a characteristic pharmacological profile that distinguishes it from other 5-HT receptors, but so far, no selective 5-HT_6 receptor ligands have been described. We attempted to identify specific interactions between the 5-HT_6 receptor and nonselective ligands to allow the adaptation of existing G protein-coupled receptor models to the binding site of the 5-HT_6 receptor. Eventually, such models could be used to suggest structural modifications that might improve ligand affinity and selectivity. Several groups have proposed three-dimensional models of the ligand binding site of other G protein-coupled 5-HT receptors (13, 36–38). As a first step, we looked for characteristic differences in the amino acid sequence of the 5-HT_6 receptor compared with other 5-HT receptors in positions that had been suggested to form part of the ligand binding site.

One of the interactions of 5-HT with the 5-HT_{2A} receptor suggested by Hibert *et al.* (13) was a hydrogen bond between a serine residue in transmembrane region IV and the indole-nitrogen (N1) of 5-HT. This serine residue is conserved in the majority of cloned mammalian 5-HT receptors, with the exception of the 5-HT_{1A} receptors (glycine), 5-HT_4 receptor (proline), and 5-HT_6 receptors (alanine). We tested whether substitution of Ala154 by serine (i.e., the introduction of a new hydroxyl group at this position) would allow the formation of a new hydrogen bond, thus changing the affinity or agonist activity of 5-HT_6 receptor ligands. However, neither 5-HT- or LSD-induced adenylyl cyclase stimulation nor the affinities of a range of compounds tested in [^{3}H]LSD binding assays were altered. This postulated interaction is apparently not possible in the case of the 5-HT_6 receptor. Whether it actually takes place in the 5-HT_{2A} or any other serotonin

TABLE 2

Change in binding energy caused by the mutation T196A

The change in free energy [$\Delta(\Delta G) = R\,T\,\ln(EC_{50(T196A)}/EC_{50(WT)})$] for the interaction of 5-HT and LSD with wild-type 5-HT$_6$ receptor and mutant T196A was calculated from the EC_{50} values determined in adenylyl cyclase assays and from the K_i or K_d values measured in [^{3}H]LSD binding assays for all ligands that showed significant changes in their affinities.

	Adenylyl cyclase stimulation			
	$EC_{50\,(WT)}$	$EC_{50(T196A)}$	$\frac{EC_{50(T196A)}}{EC_{50(WT)}}$	$\Delta(\Delta G)$
	nM			*kJ/mol*
5-HT	74	540	7.3	5.1
LSD	16	290	18.1	7.5
	[^{3}H]-LSD binding assays			
	$K_{i(d)(WT)}$	$K_{i(d)(T196A)}$	$\frac{K_{i(d)(T196A)}}{K_{i(d)\,(WT)}}$	$\Delta(\Delta G)$
	nM			*kJ/mol*
5-HT	168.2	992.5	5.9	4.6
[^{3}H]LSD	1.9	32.0	16.8	7.3
Lisuride	10.9	372.7	34.2	9.1
Ergotamine	2.4	38.6	16.1	7.2
Methysergide	289.8	41.0	0.14	−5.0
Mesulergine	2662.9	725.0	0.27	−3.4

WT, wild-type.

receptor has not yet been experimentally verified to our knowledge.

In contrast, removal of a potential hydrogen bond forming site in transmembrane helix five of the 5-HT$_6$ receptor by changing Thr196 to alanine (the residue present in most other mammalian 5-HT receptors) selectively reduced the affinity of the natural agonist 5-HT (a N1-unsubstituted indoleamine) and that of several N1-unsubstituted ergolines (LSD, lisuride, ergotamine) while not affecting the affinity of the N1-methylated compound metergoline and increasing the affinity of the N1-methylated ergolines mesulergine and methysergide. The potency of 5-HT and LSD in cAMP accumulation experiments was reduced by the same factor as the affinity determined in binding experiments. The change in free energy [$\Delta(\Delta G)$] for the interaction with 5-HT calculated from the ratio of either the EC_{50} values (5.1 kJ/mol) or K_i values (4.6 kJ/mol) was less than that for LSD (7.5 and 7.3 kJ/mol, respectively). The other N1-unsubstituted ergolines tested in [^{3}H] LSD binding assays, ergotamine (7.2 kJ/mol) and lisuride (9.1 kJ/mol), also showed larger changes in their free energy than 5-HT. However, all values were in the range expected for hydrogen bonds (2.1–7.5 kJ/mol = 0.5–1.8 kcal/mol) (39). This is consistent with the existence of a hydrogen bond between the indole N of 5-HT and N1-unsubstituted ergoline compounds and the hydroxyl group of Thr196 in the wild-type 5-HT$_6$ receptor (Fig. 7). The efficacy of adenylyl cyclase coupling was not affected by exchanging Thr196 for alanine because the maximum levels of adenylyl cyclase stimulation obtained for this mutant and wild-type receptor were similar and the partial agonist character of LSD was unchanged. The decreased potency of both 5-HT and LSD observed in the cAMP accumulation experiments with mutant T196A presumably is a direct consequence of the reduction in binding affinity that is detected in the [^{3}H] LSD binding assays. The expression levels reached for wild-type and mutant receptors were similar, so effects due to changes in the ratio of receptor to G proteins can be excluded. The affinities of metergoline and those of several antagonists with nonergoline structures were not significantly decreased. This shows that the overall structure of the receptor has been well preserved and the observed reduction of the affinity of 5-HT and N-1 unsubstituted ergolines must be due to the elimination of a specific interaction of the side chain of Thr196 and these ligands. The increased affinity of mesulergine and methysergide could be the result of the elimination of an unfavorable steric interaction between the methyl group in the N1 position of these ligands and the hydroxyl and methyl group of Thr196 in the wild-type receptor that are removed in the T196A mutant (Fig. 7). Metergoline may bind to the receptor in a slightly different orientation that is not influenced by the size of the side chain of residue 196.

The hydrogen bond that can be formed between the N1 of ergoline compounds and the hydroxyl group of Thr196 is analogous to the interaction of these compounds with the amino acid present in the corresponding position in human, pig, and squirrel monkey 5-HT$_{2A}$ receptor (Ser242) (28–32). All other cloned mammalian 5-HT receptors, including the rat 5-HT$_{2A}$ receptor, contain an alanine residue at this position (Fig. 1). N1-Unsubstituted ergolines and tryptamines have a higher affinity for human, pig, and squirrel monkey 5-HT$_{2A}$ receptors because they can form a hydrogen bond with the serine residue present in these species. N1-Substituted ergolines have a higher affinity for the rat 5-HT$_{2A}$ receptor and lower affinity for human, pig, and squirrel monkey 5-HT$_{2A}$ receptors, presumably because of unfavorable steric interactions with the hydroxyl group present in the serine-containing species subtypes. Replacement of the serine residue present in the human 5-HT$_{2A}$ receptor by alanine results in a pharmacology similar to that of the rat receptor, and replacement of the alanine in the rat sequence by serine did the reverse (28, 29). This position also contributes to the selectivity of ligands between the human 5-HT$_{2A}$ receptor and the human 5-HT$_{2C}$ receptor (33).

Among 5-HT receptors, the interaction between agonists

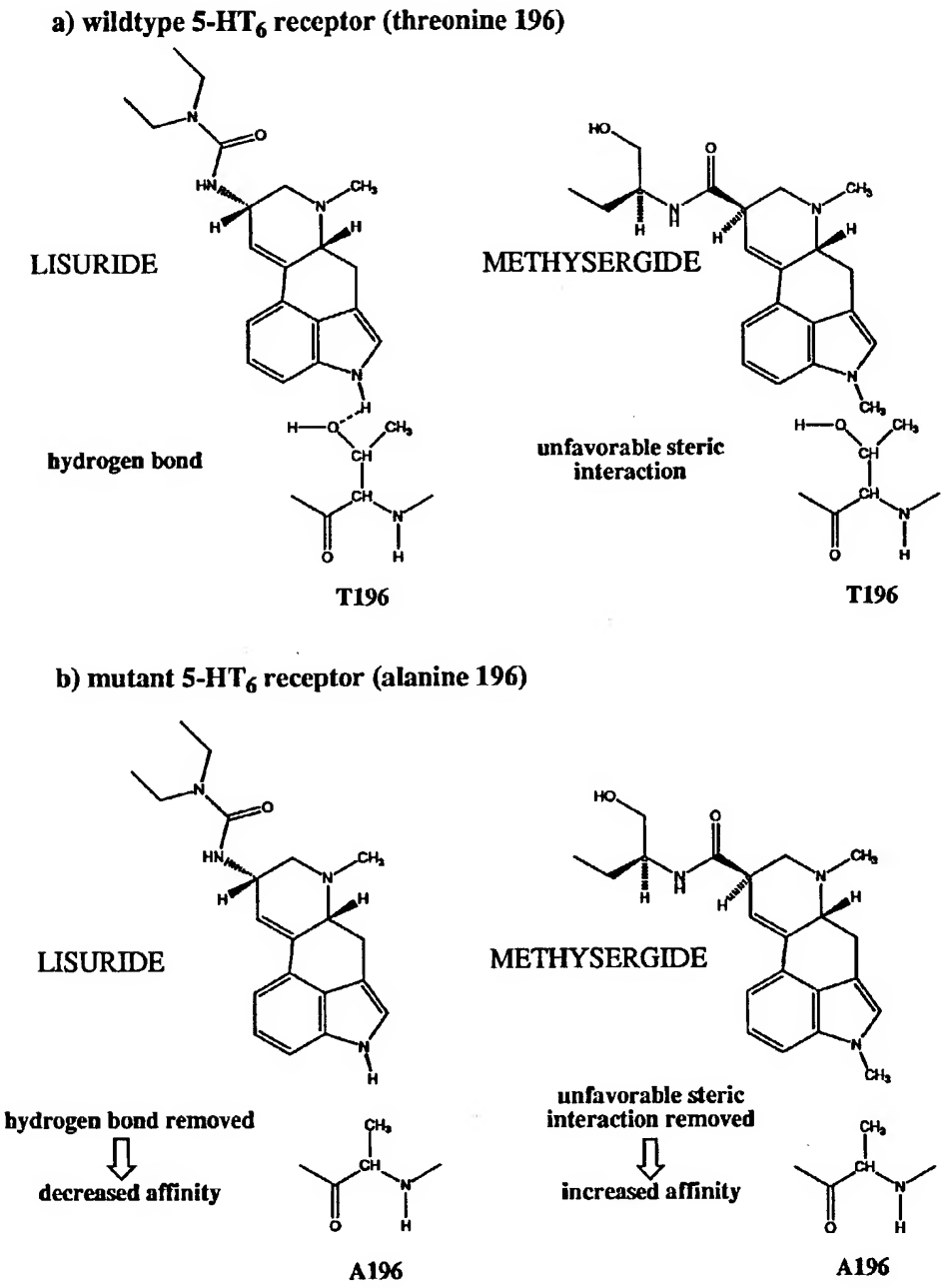

Fig. 7. Proposed interactions of a N1-unsubstituted ergoline (lisuride) and a N1-methylated ergoline (methysergide) with the side chain of residue 196 in wild-type 5-HT_6 receptor and mutant T196A.

and the second hydrogen bonding site in transmembrane region V is unique to the 5-HT_6 receptor and the species subtypes of the 5-HT_{2A} receptor mentioned above. In contrast, all 5-HT receptors cloned to date contain either a serine or a threonine residue one helix turn (three residues) closer to the extracellular surface (Ser193 in the 5-HT_6 receptor). Mutation of the corresponding residue in the 5-HT_{1A} receptor (a threonine) to alanine decreased the affinity and agonist activity of 5-HT (27). This hydrogen bond with the 5-hydroxyl-group of the indole ring of 5-HT may thus be present in the binding site of all 5-HT receptors. In the related cationic amine receptors (i.e., α_{1A}-, α_{2A}-, and β_2-adrenergic receptors; D_1 and D_2 dopamine receptors; and H_1 and H_2 histamine receptors), both positions are occupied by potential hydrogen bond-forming residues, and in most cases, both positions seem to contribute to the ligand binding site. Of particular interest with respect to our results is the suggestion that the asparagine residue present at the position corresponding to Thr196 in the histamine H_1 receptor may interact with the τ-nitrogen of the imidazole ring of histamine (24). This τ-nitrogen can assume the same relative position to the positively charged ω-nitrogen of histamine as the indole (N1) nitrogen of 5-HT to the ω-nitrogen of 5-HT. The histamine H_2 receptor contains a threonine residue at this position, and mutation of this residue also affects ligand binding and agonist potency (23).

The combination of site-directed mutagenesis (guided by knowledge obtained for related receptors) with a series of

related ligands differing in a particular structural feature has allowed the identification of a specific interaction between Thr196 in transmembrane region V of the 5-HT$_6$ receptor and the indole nitrogen of N1-unsubstituted ergolines and tryptamines. These results provide information that can be used to improve models of 5-HT$_6$ receptor/ligand interactions and may contribute to the design of selective drugs for this receptor type.

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PERSPECTIVE

Why Mice Are Neither Miniature Humans nor Small Rats: A Cautionary Tale Involving 5-Hydroxytryptamine-6 Serotonin Receptor Species Variants

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Serotonin [5-hydroxytryptamine (5-HT)] is a biogenic amine neurotransmitter that modulates a host of important biological processes, such as mood, cognition, perception, feeding behavior, smooth muscle contractility, and platelet aggregation. The myriad actions of 5-HT are mediated by one or more of at least seven subtypes of receptors, all but one of which (i.e., the 5-HT_3 receptor) are members of the G protein-coupled receptor superfamily (Kroeze et al., 2002) (see phylogram, Fig. 1). Receptors that are closer on the phylogram are more likely to share pharmacological properties than receptors that are farther apart (distant relatives). One of the most enigmatic 5-HT receptors, the 5-HT_6 receptor, was cloned in 1993 from a rat striatal cDNA library taken from sequence homology with existing 5-HT receptors and found to be expressed in the striatum, olfactory tubercule, cortex, and hippocampus (Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995). The human (Kohen et al., 1996) and mouse (Kohen et al., 2001) 5-HT_6 receptors were subsequently cloned with the human version shown to be pharmacologically similar to the rat (Kohen et al., 1996). Shortly after the discovery of 5-HT_6 receptors, Roth et al. (1994) reported that a large number of typical and atypical antipsychotic drugs bound with unexpectedly high affinity (K_i values of <20 nM) to cloned 5-HT_6 receptors. The expression of 5-HT_6 receptors in limbic areas and the basal ganglia, and the selective labeling of 5-HT_6 receptors in rat brain with [^{3}H]clozapine (Glatt et al., 1995), strongly implicated 5-HT_6 receptors in at least some of the actions of antipsychotic drugs.

More recently, 5-HT_6 receptors have been demonstrated to regulate central cholinergic neurotransmission. In this regard, the administration of the 5-HT_6 receptor-selective antagonist Ro 04-6790 reversed scopolamine-induced rotation in 6-hydroxydopamine–lesioned rats (Bourson et al., 1998). Additionally, Rogers and Hagan (2001) and Woolley et al. (2001) found that either 5-HT_6 receptor antisense oligonucleotides or 5-HT_6 receptor-selective inhibitors enhanced retention by rats of the learned platform position in the Morris water maze. These data suggest that 5-HT_6 receptor antagonists might boost cholinergic neurotransmission and reduce the cognitive impairments experienced by patients with dementia or schizophrenia. Intriguingly, Tsai et al. (1999) determined that the 267C allele of the 5-HT_6 receptor is a significant risk factor for Alzheimer's disease. Taken together, these findings indicate that 5-HT_6 antagonists might prove useful in treating a number of common illnesses, including dementia and schizophrenia.

The now-classic approach for validating 5-HT_6 receptors as molecular targets for therapeutics is to construct a 5-HT_6 knockout mouse and to characterize its phenotype. As Hirst et al. (2003) discovered, however, it is unlikely that 5-HT_6 knockout mice will be useful for validating the 5-HT_6 receptor as a therapeutic target because of pronounced and unexpected species differences in both receptor regional distribution and pharmacology. It is now widely appreciated that slight differences in rodent and human amino acid sequences can lead to unexpectedly large differences in the pharmacology of the receptors, with potentially disastrous effects for drug-discovery efforts. What has not been clearly documented until the Hirst et al. study (2003), however, is that mouse receptors could be significantly different from rat receptors.

In the article published in this issue of *Molecular Pharmacology*, Hirst et al. (2003) elegantly demonstrate that the mouse 5-HT_6 receptor is, in nearly every respect, distinct from rat and human 5-HT_6 receptors. They investigated these differences because of preliminary studies in which they were unable to quantify mouse 5-HT_6 receptors with a highly selective radioligand ([^{125}I]SB-258585) that labels both rat and human 5-HT_6 receptors. In addition to species differences in the binding of drugs to 5-HT_6 receptors, they found differences in the regional expression of 5-HT_6 receptors. Thus, quantitative polymerase

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; Ro 04-6790, 4-amino-*N*-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulfonamide; SB-258585, 4-iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulfonamide.

chain reaction studies demonstrated that the mouse 5-HT_6 receptor mRNA was at least 10-fold less abundant than the rat or human 5-HT_6 receptor mRNAs in every brain region examined. Surprisingly, whereas 5-HT_6 receptor mRNA and radioligand binding activity was enriched in the basal ganglia of rat and human brain, there was no such enrichment in the mouse brain.

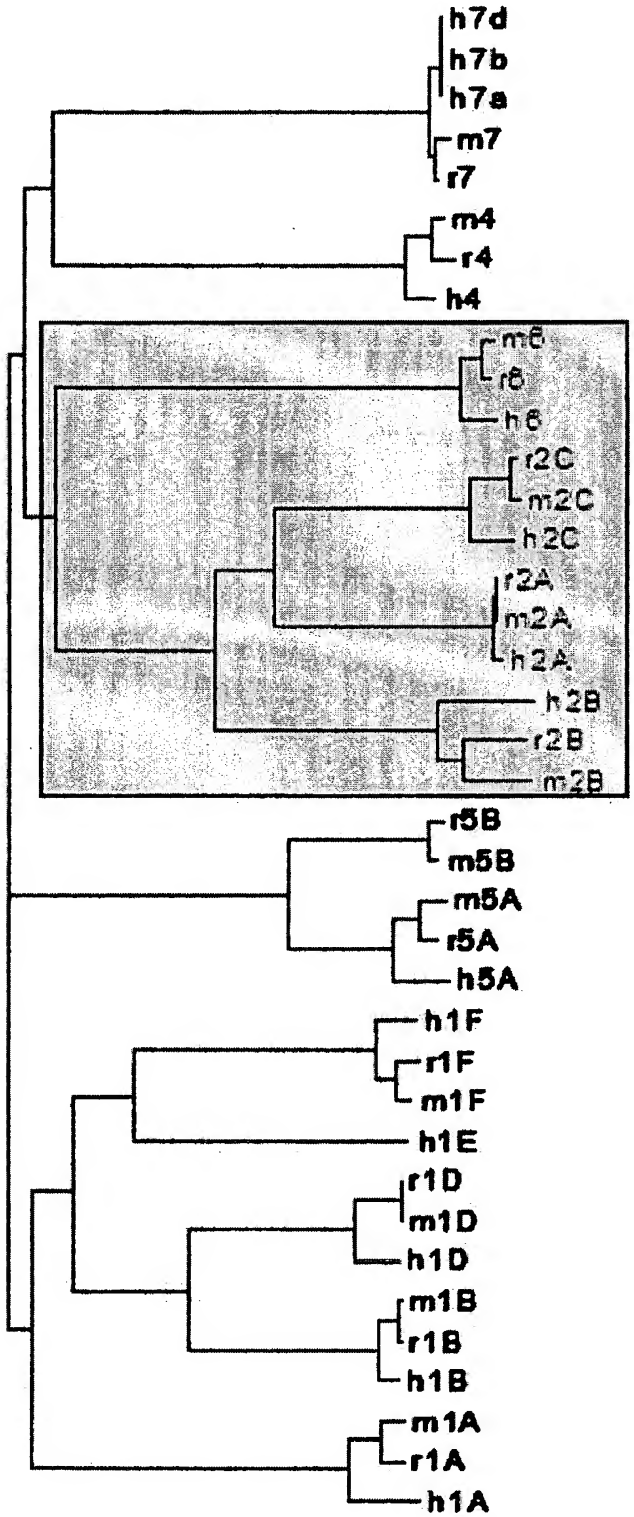

Fig. 1. Phylogram of mouse, rat, and human 5-HT G protein-coupled receptors shows that 5-HT_6 receptors are most similar in amino acid sequence to 5-HT_2 family receptors. Multiple pairwise alignments were performed, and the phylogram was produced using the AlignX module of the Vector NTI Suite (Informax, Frederick, MD).

Additionally, via a combination of site-directed mutagenesis and molecular modeling studies, Hirst et al. (2003) describe the presumed molecular and atomic reasons for the peculiar mouse 5-HT_6 pharmacology. Two amino acids—Tyr188 (in helix 5, which is Phe188 in rats and humans) and Ser290 (in helix 6 which is Asn290 in rats and humans)—were found to account for the bulk of the differences in pharmacology. A nice feature of the study is the parallel inclusion of elegant modeling studies of the various ligands used. Hirst et al. (2003) use this model to present a plausible molecular rationale for the differential interactions of various 5-HT_6 receptor-selective ligands with human, rat, and mouse 5-HT_6 receptors.

The findings described by Hirst et al. (2003) have important implications for drug discovery. Because the mouse 5-HT_6 receptor is distinct *in nearly every way* from the human (and rat) 5-HT_6 receptor, the results force us to question the use of knockout mice in a wholesale fashion to provide validated molecular targets for drug discovery. Their studies strongly imply that before a knockout mouse is accepted as a validated model for a particular human disease, the molecular target needs to be demonstrated to have a pharmacology, regional tissue distribution, and abundance similar to the human homolog. Therefore, this study stands as an important reminder to us all that mice are not miniature humans and, sometimes, not even small rats.

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